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| <b>(21) International Application Number:</b> PCT/US96/05434<br><b>(22) International Filing Date:</b> 19 April 1996 (19.04.96)<br><br><b>(30) Priority Data:</b><br>08/424,995                   19 April 1995 (19.04.95)                   US<br>08/483,959                   7 June 1995 (07.06.95)                   US<br><br><b>(71) Applicant:</b> ACORDA THERAPEUTICS [US/US]; 145 West 58th Street #8J, New York, NY 10019 (US).<br><b>(72) Inventor:</b> SCHACHNER, Melitta; Ekkehardstrasse 8, CH-8006 Zurich (CH).<br><b>(74) Agents:</b> JACKSON, David, A. et al.; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).  |           | <b>(81) Designated States:</b> AL, AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| <b>(54) Title:</b> CNS NEURITE OUTGROWTH MODULATORS, AND COMPOSITIONS, CELLS AND METHODS EMBODYING AND USING SAME<br><br><b>(57) Abstract</b><br><p>The invention features a method for promoting neural growth <i>in vivo</i> in the mammalian central nervous system by administering a neural cell adhesion molecule which can overcome inhibitory molecular cues found on glial cells and myelin to promote neural growth. Also featured active fragments, cognates, congeners, mimics, analogs, secreting cells and soluble molecules thereof, as well as antibodies thereto, and DNA molecules, vectors and transformed cells capable of expressing them. The invention also includes transgenic mouse lines expressing a neural adhesion molecule in differentiated astrocytes, and cells and tissues derived therefrom. The expression of the neural adhesion molecule enhances neurite outgrowth on central nervous system tissue derived from these transgenic mice. The invention also features methods for enhancing neuronal outgrowth of CNS neurons, for enhancing memory and for increasing synaptic efficacy. Also featured are methods of testing drugs which modulate the effects of the neural adhesion molecule, and assay systems suitable for such methods.</p> |           |  |

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**CNS NEURITE OUTGROWTH MODULATORS, AND COMPOSITIONS,  
CELLS AND METHODS EMBODYING AND USING SAME**

**BACKGROUND OF THE INVENTION**

**Field of the Invention**

5 This invention relates generally to the modulation of neural growth in the central nervous system, and more particularly to methods and associated agents, constructs and compositions for improving CNS neural growth. Specifically, the invention relates to the use of cellular adhesion molecules, and preferably neural cell adhesion molecules such as L1, to foster and improve such neural growth.

**Description of the Related Art**

10 The ability of neurons to extend neurites is of prime importance in establishing neuronal connections during development. It is also required during regeneration to re-establish connections destroyed as a result of a lesion.

Neurites elongate profusely during development both in the central and peripheral  
15 nervous systems of all animal species (Cajal (1928) *Degeneration and regeneration in nervous system*, Oxford University Press, London). This phenomenon pertains to axons and dendrites. However, in adults, axonal and dendritic regrowth in the central nervous system is increasingly lost with evolutionary progression.

In the peripheral nervous system, after infliction of a lesion, axons of all vertebrate  
20 species are able to regrow (Cajal (1928); Martini (1994) *J. Neurocytol.* 23:1-28). However, in mammals, neurite regrowth following damage is limited to neuritic sprouting. Regrowth of neuronal processes is, however, possible in lower vertebrate species (Stuermer et al. (1992) *J. Neurobiol.* 23:537-550). In contrast, in  
the central nervous system, most, if not all neurons of both higher and lower  
25 vertebrate adults possess the potential for neurite regrowth (Aguayo (1985) "Axonal regeneration from injured neurons in the adult mammalian central nervous system." In: *Synaptic Plasticity* (Cotman, C.W., ed.) New York. The Guilford Press, pp. 457-484.)

Glial cells are the decisive determinants for controlling axon regrowth. Mammalian glial cells are generally permissive for neurite outgrowth in the central nervous system during development (Silver et al. (1982) *J. Comp. Neurol.* 210:10-29; Miller et al. (1985) *Develop. Biol.* 111:35-41; Pollerberg et al. (1985) *J. Cell. Biol.* 101:1921-1929) and in the adult peripheral nervous system (Fawcett et al. (1990) *Annu. Rev. Neurosci* 13:43-60). Thus, upon infliction of a lesion, glial cells of the adult mammalian peripheral nervous system can revert to some extent to their earlier neurite outgrowth-promoting potential, allowing them to foster regeneration (Kalderon (1988) *J. Neurosci Res.* 21:501-512; Kliot et al. "Induced regeneration of dorsal root fibres into the adult mammalian spinal cord," In: *Current Issues in Neural Regeneration*, New York, pp. 311-328; Carlstedt et al. (1989) *Brain Res. Bull.* 22:93-102). Glial cells of the central nervous system of some lower vertebrates remain permissive for neurite regrowth in adulthood (Stuermer et al. (1992) *J. Neurobiol.* 23:537-550). In contrast, glial cells of the central nervous system of adult mammals are not conducive to neurite regrowth following lesions.

Several recognition molecules which act as molecular cues underlying promotion and/or inhibition of neurite growth have been identified (Martini (1996). Among the neurite outgrowth promoting recognition molecules, the neural cell adhesion molecule L1 plays a prominent role in mediating neurite outgrowth (Schachner (1990) *Seminars in the Neurosciences* 2:497-507). L1-dependent neurite outgrowth is mediated by homophilic interaction. L1 enhances neurite outgrowth on L1 expressing neurites and Schwann cells, and L1 transfected fibroblasts (Bixby et al. (1982) *Proc. Nat'l Acad. Sci. U.S.A.* 84:2555-2559; Chang et al. (1987) *J. Cell. Biol.* 104:355-362; Lagenaur et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:7753-7757; Seilheimer et al. (1988) *J. Cell. Biol.* 107:341-351; Kadmon et al. (1990a) *J. Cell. Biol.* 110:193-208; Williams et al. (1992) *J. Cell. Biol.* 119:883-892). Expression of L1 is enhanced dramatically after cutting or crushing peripheral nerves of adult mice (Nieke et al. (1985) *Differentiation* 30:141-151; Martini et al. (1994a) *Glia* 10:70-74). Within two days L1 accumulates at sites of contact between neurons and Schwann cells being concentrated mainly at the cell surface

of Schwann cells but not neurons (Martini et al. (1994a)). Furthermore, the homophilic binding ability of L1 is enhanced by molecular association with the neural cell adhesion molecule N-CAM, allowing binding to occur through homophilic assistance (Kadmon et al. (1990a); Kadmon et al. (1990b) *J. Cell Biol.* 110:209-218 and 110:193-208; Horstkorte et al. (1993) *J. Cell. Biol.* 121:1409-1421). Besides its neurite outgrowth promoting properties, L1 also participates in cell adhesion (Rathjen et al. (1984) *EMBO J.* 3:1-10; Kadmon et al. (1990b) *J. Cell. Biol.* 110:209-218; Appel et al. (1993) *J. Neurosci.*, 13:4764-4775), granule cell migration (Lindner et al. (1983) *Nature* 305:427-430) and myelination of axons (Wood et al. (1990) *J. Neurosci* 10:3635-3645).

L1 consists of six immunoglobulin-like domains and five fibronectin type III homologous repeats. L1 acts as a signal transducer, with the recognition process being a first step in a complex series of events leading to changes in steady state levels of intracellular messengers. The latter include inositol phosphates,  $\text{Ca}^{2+}$ , pH and cyclic nucleotides (Schuch et al. (1990) *Neuron* 3:13-20; von Bohlen und Hallbach et al. (1992) *Eur. J. Neurosci.* 4:896-909; Doherty et al. (1992) *Curr. Opin. Neurobiol.* 2:595-601) as well as changes in the activities of protein kinases such as protein kinase C and  $\text{pp60}^{\text{src}}$  (Schuch et al. (1990) *Neuron* 3:13-20; Atashi et al. (1992) *Neuron* 8:831-842). L1 is also associated with a casein type II kinase and another unidentified kinase which phosphorylates L1 (Sadoul et al. (1989) *J. Neurochem* 328:251-254). L1-mediated neurite outgrowth is sensitive to the blockage of L type  $\text{Ca}^{2+}$  channels and to pertussis toxin. These findings indicate the importance of both  $\text{Ca}^{2+}$  and G proteins in L1-mediated neurite outgrowth (Williams et al. (1992) *J. Cell. Biol.* 119:883-892). L1 is also present on proliferating, immature astrocytes in culture and neurite outgrowth is promoted on these cells far better than on differentiated, L1 immunonegative astrocytes (Saad et al. (1991) *J. Cell. Biol.* 115:473-484). *In vivo*, however, astrocytes have been found to express L1 at any of the developmental stages examined from embryonic day 13 until adulthood (Bartsch et al. (1989) *J. Comp. Neurol* 284:451-462; and unpublished data).

Given the capability of L1 to promote neurite outgrowth, it is pertinent to investigate whether astrocytic expression of L1 and other members of the immunoglobulin superfamily to which L1 belongs, may overcome potentially inhibitory molecular cues reported to be present on glial cells and myelin in the adult central nervous system (Schachner et al., *Perspectives in Developm. Neurobiol.* in Press; Schwab et al. (1993) *Ann. Rev. Neurosci.* 16:565-595). This is of particular relevance to the development of effective strategies for the treatment of debilitation caused by the malformation of or injury to neural tissues of the CNS, and it is toward such objectives that the present invention is directed.

10

### SUMMARY OF THE INVENTION

In accordance with the present invention, an agent and corresponding methods are disclosed for the modulation of neural growth and particularly, such growth as can be promoted in the compartment of the central nervous system (CNS), and specifically, in myelinated nerve tissue. The agents of the present invention are notable in their ability to promote such neural growth in an environment that has been traditionally viewed as inhibitory to the growth promoting stimulus of known neurite outgrowth factors. Specifically, this inhibitory environment includes inhibitory molecular cues which are present on glial cells and myelin the central nervous system.

20 The agents of the present invention are broadly selected from a group of cell adhesion molecules, and more preferably neural cell adhesion molecules. Most preferably, the agents of the present invention are selected from the group of molecules belonging to the immunoglobulin superfamily, and particularly to those members that mediate  $\text{Ca}^{2+}$ -independent neuronal cell adhesion, of which L1, N-CAM and myelin-associated glycoprotein are particular members. Other cell adhesion molecules which may also influence CNS neural growth include laminin, fibronectin, N-cadherin, BSP-2 (mouse N-CAM), D-2, 224-1A6-A1, L1-CAM, NILE, Nr-CAM, TAG-1 (axonin-1), Ng-CAM and F3/F11.

- In a further aspect of the present invention, the agents of the invention belong to a new family referred to herein as the L1 family of neural recognition molecules. This family includes L1, NgCAM, neurofascin, *Drosophila* neuroglian, zebrafish L1.1 and L1.2, and others. This group of agents all demonstrate the Ig-like domains and FN-like repeats that are characteristic of L1, and in this connection, exhibit a remarkable colinearity, a high degree of N-glycosidically linked carbohydrates, which include the HNK-1 carbohydrate structure, and a pattern of protein fragments comprising a major 185 kD band and smaller bands of 165 and 125 kD.
- 10 The agents of the present invention also include fragments of cell adhesion molecules and cognate molecules, congeners and mimics thereof which modulate neurite growth in the CNS. In particular, the agents include molecules which contain structural motifs characteristic of extracellular matrix molecules, in particular the fibronectin type III homologous repeats and immunoglobulin-like domains. Preferably, these structural motifs include those structurally similar to 15 fibronectin type III homologous repeats 1-2, and immunoglobulin-like domains I-II, III-IV and V-VI.
- The invention extends to methods of promoting and enhancing neural regeneration *in vivo*, and to the corresponding genetic constructs, such as plasmids, vectors, transgenes, and the like, and to pharmaceutical compositions, all of which may be 20 used to accomplish the objectives of such methods. More specifically, the agents of the present invention may be prepared as vectors or plasmids, and introduced into neural cells located at a site in the CNS where regeneration is needed, for example, by gene therapy techniques, to cause the expression of an agent of the 25 present invention and to thereby promote the requisite neural growth. Another strategy contemplates the formulation of one or more of the appropriate agents in a composition that may likewise be directly delivered to a CNS site, as by parenteral administration. As certain of the agents, such as L1, have demonstrated homophilic binding, the administration of such a composition may serve the purpose of

inhibiting rather than promoting neural growth. This effect may be desirable in particular instances where unwanted or uncontrolled growth may occur or is occurring, and therefore the invention extends to this use as well.

Correspondingly, the capability of the agents to engage in homophilic binding  
5 renders antagonists to the agents, including antibodies thereto, capable of acting as agonists, and thereby participating in the promotion of neural growth and regeneration. Thus, the invention extends to the preparation of appropriate constructs and compositions containing the antibodies to the agents, for the therapeutic purposes set forth herein. Also, and as demonstrated later on herein,  
10 antibodies to L1, for example, may serve as part of a drug discovery assay or the like, to identify further agents that may possess activity and utility both diagnostic and therapeutic, in accordance with the present invention. Particularly, and as illustrated later on herein with reference to the isolation and characterization of CHL1, an L1 analog, antibodies such as polyclonal antibodies, may be used to  
15 identify further members of the L1 CAM family, and the invention accordingly extends to such CAM members as are isolated by use of such antibodies.

The invention also covers diagnostic applications, where for example, it is desirable to assess the potential for or actual development of CNS neural growth by the detection and measurement of the presence, amount or activity of one or more of  
20 the agents of the invention. Likewise, and as described hereinafter, the invention also extends to assays, including drug discovery assays, that capitalize on the activity of the agents of the present invention in the modulation of CNS neural growth. For example, prospective drugs may be tested for CNS neural growth modulation by means of an assay containing an agent of the invention, or a cell  
25 line or culture developed in conjunction herewith may serve as the assay system.

Briefly, the present invention also features transgenic mouse lines expressing a neural adhesion molecule in differentiated astrocytes and glial cells, and cells and tissues derived therefrom. In particular, the neural adhesion molecule is L1. The



astroglial L1 expression enhances neurite outgrowth on central nervous system tissue derived from these transgenic mice.

Also as discussed, the invention features methods for enhancing neuronal outgrowth of CNS neurons, for enhancing memory and for increasing synaptic efficacy, as  
5 measured by stabilization of long term potentiation, and other similarly useful methods. Also featured are methods of testing drugs and other manipulations which modulate the effects of the neural adhesion molecule, and assay systems suitable for such methods.

Accordingly, it is a principal object of the present invention to provide a transgenic  
10 mammal, the glial cells of which express an exogenous neural adhesion molecule.

A further object of the invention is to provide a cell culture containing the glial cells of the transgenic mammal.

Yet another object of the invention is to provide a cell culture system containing lesioned or unlesioned optic nerves or other parts of the nervous system of the  
15 transgenic mammal.

Still a further object of the invention is to provide a method for enhancing neuronal outgrowth of CNS neurons, which includes culturing the neurons on the glial cell culture system.

A further object of the invention is to provide a method for enhancing neuronal  
20 outgrowth of CNS neurons, which includes culturing the neurons on the optic nerve or other parts of the nervous system placed in the cell culture system.

A still further object of the invention is to provide a method for enhancing neuronal outgrowth of CNS neurons, which includes the secretion of neural adhesion molecule by implanted cells.

Another object of the invention is to provide a method for enhancing memory, which includes administering to the brain of a mammal in need of memory enhancement, an amount of the cells of the glial cell culture system effective to enhance the memory of the mammal.

- 5 Yet another object of the invention is to provide a method for enhancing memory, including administering to the brain of a mammal in need of memory enhancement, an amount of the cells of the optic nerve or other parts of the nervous system placed in the cell culture system effective to enhance the memory of the mammal.

- 10 A still further object of the invention is to provide a method for enhancing memory, including delivering to the glial cells of the brain of a mammal in need of such memory enhancement, a vector which allows for the expression of a neural adhesion molecule in the glial cells.

A further object of the invention is to provide a method for enhancing memory, which includes the secretion of neural adhesion molecule by implanted cells.

- 15 Another object of the invention is to provide a method for increasing synaptic efficacy in the CNS of a mammal in need of such an increase, including administering to the brain of the mammal, an amount of the cells of the glial cell culture system effective to increase synaptic efficacy in the brain of the mammal.

- 20 Yet a further object of the invention is to provide a method for increasing synaptic efficacy in the CNS of a mammal in need of such an increase, including administering to the brain of the mammal, an amount of the cells of the optic nerve or other parts of the nervous system placed in the cell culture system effective to increase synaptic efficacy in the brain of the mammal.

- ~~A still further object is to provide a method for increasing synaptic efficacy in the~~  
25 CNS of a mammal in need of such an increase, which includes delivering to the

glial cells of the brain of a mammal in need of such enhancement, a vector which allows for the expression of a neural adhesion molecule in the glial cells.

A further object of the invention is to provide a method for increasing synaptic efficacy, which includes the secretion of neural adhesion molecule by implanted  
5 cells.

Another object of the invention is to provide a method of testing the ability of a drug or other entity to modulate the activity of a neural adhesion molecule, which includes adding CNS neurons to the glial cell culture system; adding the drug under test to the cell culture system; measuring the neuronal outgrowth of the CNS  
10 neurons; and correlating a difference in the level of neuronal outgrowth of cells in the presence of the drug relative to a control culture to which no drug is added to the ability of the drug to modulate the activity of the neural adhesion molecule.

Another object of the invention is to provide a method of testing the ability of a drug or other entity to modulate the activity of a neural adhesion molecule which  
15 includes adding CNS neurons to the optic nerve or other parts of the nervous system cell culture system; adding the drug under test to the cell culture system; measuring the neuronal outgrowth of the CNS neurons; and correlating a difference in the level of neuronal outgrowth of cells in the presence of the drug relative to a control culture to which no drug is added to the ability of the drug to modulate the  
20 activity of the neural adhesion molecule.

Yet another object of the invention is to provide an assay system for screening drugs and other agents for ability to modulate the production of a neural adhesion molecule, which includes the glial cell culture system; and CNS neurons added to the cell culture system.

25 A further object of the invention is to provide an assay system for screening drugs and other agents for ability to modulate the production of a neural adhesion

molecule, which includes culturing the glial cell culture system inoculated with a drug or agent; adding CNS neurons to the cell culture system; and examining neuronal outgrowth to determine the effect of the drug thereon.

- Yet another object of the invention is to provide an assay system for screening  
5 drugs and other agents for ability to modulate the production of a neural adhesion molecule, which includes culturing the optic nerve or other parts of the nervous system in the cell culture system inoculated with a drug or agent; adding CNS neurons to the cell culture system; and examining neuronal outgrowth to determine the effect of the drug thereon.
- 10 Another object of the invention is to provide an assay system for screening drugs and other agents for ability to modulate the production of a neural adhesion molecule, which includes inoculating a culture of CNS neurons with a drug or agent; adding a soluble neural adhesion molecule; and examining neuronal outgrowth to determine the effect of the drug thereon.
- 15 Other objects and advantages will become apparent to those skilled in the art from a review of the ensuing detailed description taken with reference to the following illustrative drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

- FIGURE 1 depicts the map of the GFAP-L1 chimeric transgene. A 4.05 kb mouse  
20 L1 cDNA was inserted into exon 1 of a modified GFAP gene using *Not* I linkers. In this construct, the L1 cDNA is preceded 5' by an SV40 late gene splice (V) and followed 3' by an SV40 polyadenylation signal (pA). The locations of the L1 ATG and the polyadenylation signal are indicated. Exons are shown as boxes.

- FIGURE 2 depicts a Northern blot analysis of brain RNA from different transgenic  
25 lines. 10 µg of total RNA of whole adult brain was loaded in each lane and

probed with mouse L1 cDNA. Exposure time was 3 days. Lanes 1-3, brains from different transgenic offspring (lane 1: line 3426; lane 2: line 3427; lane 3: line 3418; lane 4, brain from non-transgenic control). Note that the level of transgenic L1 mRNA (arrow) is different in the three transgenic lines, with levels being  
5 highest in line 3426, intermediate in line 3427 and lowest in line 3418. The position of 28S and 18S rRNA is shown on the right margin.

FIGURE 3 depicts the localization of L1 mRNA in adult unlesioned (A, C and E) and lesioned (15 days after the lesion, B and D) optic nerves from non-transgenic (A, B and E) and transgenic mice (C and D) of line 3426 by *in situ* hybridization.  
10 In wild type animals, L1 mRNA is detectable only in neuronal cells of the retina but not in the glial cells of the optic nerve (A and B). In transgenic animals, cells containing L1 transcripts are visible in the optic nerve (C and D). The density of L1 positive cells is highest in the unmyelinated proximal part of the nerve. The density of L1 mRNA positive cells in the nerve is slightly increased after a lesion  
15 (compare C and D). In the optic nerve, the distribution of cells expressing L1 (C and D) is similar to that of cells expressing GFAP (E). Scale bar in E: 100  $\mu$ m (for A to E).

FIGURE 4 depicts the double immunofluorescence microscopic localization of L1 (A and B) and GFAP (C) in unlesioned (A and C) and lesioned (28 days after the  
20 lesion, B) optic nerves from adult transgenic (line 3426, A and B) and wild type (C) animals. L1 immunoreactivity in optic nerves from transgenic animals is significantly increased after a lesion (compare A and B). The pattern of L1 immunoreactivity in lesioned transgenic nerves is similar to the pattern of GFAP immunostaining in unlesioned wild type nerves. L1 positive unmyelinated retinal  
25 cell ganglion axons are present in unlesioned wild type nerve (A). Scale bar in C: 50  $\mu$ m (For A to C).

FIGURE 5 depicts the double immunofluorescence microscopic localization of L1 (A and D) and GFAP (B and E) in cultured astrocytes from transgenic animals of

line 3426 (A, B and C) and wild type animals (D, E and F). Note that only the cells from transgenic animals express L1, whereas astrocytes from wild type animals are L1 negative. Scale bar in F:30  $\mu$ m (for A to F).

FIGURE 6 shows (A) Western blot analysis of lesioned (15 days after the lesion) and unlesioned optic nerves from transgenic and wild type animals. 25  $\mu$ g of total protein of lesioned (lanes 1, 3 and 5) or unlesioned nerves (lanes 2, 4, and 6) was loaded and detected using affinity purified polyclonal antibodies against L1. Protein extracts were made from mice of transgenic lines 3426 (lanes 1 and 2), 3427 (lanes 3 and 4) and from wild type animals (lanes 5 and 6). There is an increase in L1 expression in transgenic animals compared to non-transgenic controls. Following optic nerve lesion, an up-regulation of L1 occurred in transgenic animals, whereas the amount of L1 in wild type animals decreased. Apparent molecular weights (in kD) are shown on the left margin.

FIGURE 7 depicts examples of neurite outgrowth from mouse cerebellar neurons cultured on cryostat sections of optic nerves from wild type (A and B) and transgenic (C and D) animals (line 3426). A and C represent unlesioned optic nerves, B and D represent lesioned optic nerves. Scale bar in D:50  $\mu$ m (for A to D).

FIGURE 8 depicts and compares neurite lengths of cerebellar neurons maintained on cryostat sections of unlesioned (c) and lesioned (l) optic nerves (28 days after the lesion) from wild type (WT) and transgenic animals (lines 3426, 3427 and 3418). Note that the length of neurites on sections from transgenic animals is greater than on sections from wild type animals. In transgenic lines neurites are always longer on lesioned than on unlesioned nerves, whereas neurite lengths on unlesioned and lesioned nerves of wild type animals do not show a significant difference. Note that the neurite length correlates positively with the levels of L1 expression in different transgenic lines (see also Western blot data). Mean values  $\pm$

standard error of the mean from one representative experiment (out of 12) are shown.

- FIGURE 9 is a graph measuring neurite lengths of cerebellar neurons maintained on cryostat sections of unlesioned (c) and lesioned (l) optic nerves (28 days after the lesion) from wild type (WT) and transgenic animals without and after pre-incubation of sections with affinity purified polyclonal antibodies against L1 (anti L1) and mouse liver membranes (anti liver). Neurite lengths on nerves without pre-incubation with any antibody were taken as 100% and neurite lengths on sections of the same nerves obtained after antibody treatment were expressed in relation to this value. A significant reduction (60%) of neurite length by L1 antibodies was found on cryostat sections from transgenic animals. Numbers on the top represent the total number of nerves measured for each value. Mean values  $\pm$  standard error of the mean are from at least four independent experiments carried out in duplicate.
- FIGURE 10 depicts and compares neurite lengths of mouse cerebellar (A) or chick DRG (B) neurons on astrocytic monolayers prepared from wild type (WT) and transgenic animals (line 3426) in the absence of antibodies and after pre-incubation of sections with affinity purified polyclonal antibodies against L2 (anti L1) and mouse liver membranes (anti liver). The neurite length on astrocytes without pre-incubation with any antibody was taken as 100% and the neurite lengths on astrocyte monolayers obtained after antibody treatment are expressed in relation to this value. A significant reduction (about 40%) of neurite length is only visible on transgenic astrocytes after preincubation of the monolayers with L1 antibodies. Mean values  $\pm$  standard deviation are from at least 100 neurons from two independent experiments carried out in quadruplicate. \* indicates means that were significantly different ( $p < 0.05$ , Mann-Whitney U test) from the control (wild type or transgenic astrocytes without any antibody treatment).

FIGURE 11 demonstrates the *in vivo* regrowth of axons in the optic nerve (0-2000  $\mu$ m). 6-8 week old GFAP-L1 transgenic mice and wild type mice were crushed

intraorbitally and, after 14 days, traced with a fluorescein-labeled biotin ester to mark retinal ganglion cell axons by anterograde labeling. Each point represents one animal.

FIGURE 12 depicts *in vivo* regrowth of axons in the optic nerve (0-800  $\mu$ m). 6-8 week old GFAP-L1 transgenic mice and wild type mice were crushed intraorbitally and, after 14 days, traced with a fluorescein-labeled biotin ester to mark retinal ganglion cell axons by anterograde labeling. Each point represents one animal.

FIGURE 13 shows the effect of the injection of chicken L1 antibodies into the IMHV on percent avoidance (retention of memory) on a one-trial passive avoidance task. Each point represents a group of birds who received injections of L1 antibodies (anti-L1) (closed circles) or saline (open squares) at the time relative to training indicated. All animals were tested at 24 hours post-training (\*,  $p < 0.05$  between saline and antibody groups,  $\chi^2$ ).

FIGURE 14 comprises two graphs depicting the effect of injections of Ig I-IV and FN fragments at -30 minutes and +5.5 hours on retention of memory for passive avoidance task. All animals were tested at 24 hours post-training. The number of animals in each group is shown in the histograms (\* $p < 0.05$ ; \*\* $p < 0.005$ ).

FIGURE 15 comprises a series of graphs showing the influence of antibodies against L1 (anti-L1) on LTP in pyramidal neurons in the CA1 region of rat hippocampal slices. a, Averaged ( $n=4$ ) EPSP's recorded before and 50 minutes after (arrow) TBS at the control site not injected with antibodies. b, Averaged ( $n=4$ ) EPSPs recorded before and 50 min. after TBS (arrow) in the presence of rabbit polyclonal antibodies against mouse L1 (Rathjen et al. (1984)). c, Time-course of the EPSP initial slope before and after TBS in the presence of L1 antibodies (IgG fraction, 6.2 mg/ml  $\bigcirc$ ) or polyclonal antibodies to the immunoglobulin-like domains I-VI-recombinantly-expressed in CHO cells (Hynes (1992) *Cell*. 69:11-25) (antiserum containing approximately 1 mg/ml of specific



antibodies. ▼) and the following controls: (1) Control LTP (no antibodies. □), (2) in the presence of the IgG fraction of the polyclonal antibodies to mouse liver membranes (3.5 mg/ml, ●), which react strongly with rat hippocampal slices (Lindner et al. (1983) *Nature* 305:427-430), (3) in the presence of rabbit non-immune serum, and (4) in the presence of L1 antibodies without induction of LTP by TBS (6.2 mg/ml, ○; see also e, f). Results are expressed as means  $\pm$  S.E.M. of the EPSP initial slope i percent of the baseline values recorded during the 20 min. before TBS (n=5) slices from at least 3 animals; values for LTP's in the presence of L1 antibodies differ from the control LTP at  $p < 0.001$  for the antibodies against L1. and at  $p < 0.01$  for the antibodies to the immunoglobulin-like domains I-VI). d, Concentration-dependence of the reduction in LTP by the IgG fraction of polyclonal antibodies against L1 (6.2 mg/ml; ○) : 2 mg/ml, ●; 0.6 mg/ml, ▲; 0.06 mg/ml, □;  $p < 0.0001$ ). As a control, the results from polyclonal antibodies against liver membranes are shown (3.5 mg/ml, ■). e. Averaged (n=4) EPSP's recorded before and 60 min. after (arrow) the application of polyclonal antibodies against L1 in the absence of TBS. f. Averaged (n=4) intracellular excitatory postsynaptic currents (EPSP) recorded before and 30 min. after (arrow) the application of polyclonal antibodies against L1 in the absence of TBS.

FIGURE 16 demonstrates the influence of the immunoglobulin-like domains I-VI, polyclonal NCAM antibodies and oligomannosidic glycopeptides on LTP. a, time-course of the EPSP initial slope before and after TBS in the presence of the immunoglobulin (Ig)-like domains I-VI (216  $\mu$ g/ml; 3.2 mM; in 20 mM Tris/HCl pH 7.6 ○;  $p < 0.01$ ) and the fibronectin (FN) type III homologous repeats I-V (225  $\mu$ g/ml; 3.8 mM; in 20 mM Tris/HCl pH 7.6, ■) of L1, compared to control LTP (20 mM Tris/HCl, pH 7.6, □). b, Time-course of the EPSP initial slope before and after TBS in the presence of antibodies to NCAM (IgG fraction, 3.9 mg/ml, ▲), an antiserum against axonin-1 (●), and the following controls: (1) a non-immune rabbit serum (▲), (2) an IgG fraction of non-immune rabbit serum (3.0 mg/ml. ♦), and (3) in the presence of NCAM antibodies (3.9 mg/ml. □;  $p < 0.06$ ) without induction of LTP by TBS. c, time-course of the EPSP initial slope before and after

TBS in the presence of oligomannosidic glycopeptides (○), control glycopeptides ( ) derived from asialofetuin (both at 100  $\mu$ M), and in the absence of glycopeptides (□). Results are expressed as means  $\pm$  S.E.M. of the EPSP initial slope in percent of the baseline values recorded during the 20 min. before TBS (n=5 or 6 slices from at least 3 animals).

FIGURE 17 graphically depicts the influence of L1 antibodies and oligomannosidic carbohydrates on previously established LTP and on NMDA receptor-mediated synaptic transmission. a, Time-course of the EPSP initial slope before and after TBS in the presence of L1 antibodies applied either throughout the experiment (6.2 mg/ml; ○) or starting 10 minutes after TBS (6.2 mg/ml; ●). b, time-course of the EPSP initial slope before and after TBS in the presence of oligomannosidic carbohydrates applied either throughout the experiment (100  $\mu$ M; ○) or starting 20 minutes after TBS (100  $\mu$ M; ●). c, Averaged (n=4) NMDA receptor-dependent EPSP's recorded in the presence of CNQX (30  $\mu$ M) before and after 30 minutes (arrow) application of L1 antibodies. d, Averaged (n=4) NMDA receptor-dependent EPSP's recorded in the presence of CNQX (10  $\mu$ M) before and after (arrow) 60 minutes of application of oligomannosidic carbohydrates. Results in a and b are expressed as means  $\pm$  S.E.M. of the EPSP initial slope in percent of the baseline values recorded during the 20 min. before TBS (n=5 slices from at least 3 animals).

FIGURE 18 depicts the nucleotide sequence of the 4.43 kb cDNA insert of clone pX#2 and deduced amino acid sequence of mouse CHL1. The longest open reading frame (bp 296 to bp 3922) contains 1209 amino acids terminating with a TGA termination codon. The two hydrophobic regions representing the signal peptide (amino acids 1-24) and the transmembrane region (1082-1104) are underlined by a bar. Two arrows indicate the 5' and 3' ends of clone 311 isolated from the  $\lambda$ gt11 library. Potential sites of asparagine-linked glycosylation (Hubbard and Ivatt, 1981) are marked below the amino acid sequence with filled diamonds. The immunoglobulin (Ig)-like domains are numbered with roman numerals from I to VI below the conserved tryptophan. The characteristic cysteines are indicated by

circles. The FN-like repeats are numbered F1 to F5 and the characteristic tryptophans (missing in F1; F2: W 732, F3: W 830, F4: W 936, F5: W 1053) and tyrosines/phenylalanines (F1: Y 682, F2: Y 781, F3: F888, F4: Y 989, and missing in F5) are boxed. A bracket highlights the RGD and DGEA sequences (amino acid residues 185-187 and 555-558, respectively). Untranslated sequences are shown numbered in italics. The sequence data are available from EMBL/Genbank/DDBJ under accession number X94310.

FIGURE 19 depicts the domain structure, coding region of the bacterially expressed protein fragment, and hydrophobicity plot of mouse CHL1

- 10 (a) The diagram sketches the structural features deduced from the primary sequence of CHL1. Numbers refer to the amino acid sequence starting at the translation start site. Ig-like domains I to VI are represented by half circles (amino acid numbers refer to the cysteines forming the disulfide bridges). FN-like repeats 1 to 5 are symbolized by boxes (amino acid numbers refer to the domain boundaries). The potential sites for N-glycosylation are indicated by filled circles. Signal peptide and transmembrane region are denoted by etched boxes. (b) The bar indicates the position of the cDNA encoding the recombinant protein produced in *E. coli* (c) The hydrophobicity plot (Kyte and Doolittle, 1982) of the deduced amino acid sequence shows the characteristic features of an integral membrane protein with the putative hydrophobic signal sequence and transmembrane domain (\*). Positive values indicate hydrophobicity. Numbering of the abscissa refers to amino acid position.

FIGURE 20 shows the alignment of the intracellular domains of molecules of the L1 family. The sequences of the intracellular domains starting with the first amino acid residue after the putative transmembrane regions are aligned for mouse CHL1, mouse L1, chicken Nr-CAM, chicken Ng-CAM, chicken neurofascin, *Drosophila* neuroglia, and zebrafish L1.2. The numbers refer to the amino acid positions of CHL1 and gray boxes indicate gaps introduced in the CHL1 sequence. Identical

amino acids occurring in the majority of sequences are marked by black boxes. The three brackets (I, II and III) refer to highly conserved stretches.

FIGURE 21 is a Northern blot analysis of CHL1 and L1 mRNA in different tissues of mouse and rat

- 5 (a) Poly (A) RNA (2 $\mu$ g) from brain minus cerebellum (lanes 1,5), spinal cord (lanes 3,7), and dorsal root ganglia (lanes 4, 8) and total RNA (10  $\mu$ g) from cerebellum (lanes 2,8) of nine-day-old mice were hybridized with CHL1 (lanes 1 to 4) or L1 (lanes 5 to 8) riboprobes. Poly (A)\* RNA (1  $\mu$ g) from kidney (lane 9), spleen (lane 10), liver (lane 11), and thymus (lane 12) and poly (A)\* RNA (0.5 $\mu$ g) 10 from intestine (lane 13) and lung (lane 14) of nine-day-old mice were hybridized with the CHL1 riboprobe.
- (a) Total RNA (20  $\mu$ g) of NGF induced (lane 1) and non-induced (lane 2) PC12 cells, COS-1 cells (lane 3), and total RNA (30  $\mu$ g) of cerebellum of nine- (lane 4) and six- (lane 5) day-old rats were hybridized with the CHL1 riboprobe. RNA 15 markers are indicated at the left margins.

FIGURE 22 demonstrates the specificity of polyclonal antibodies against CHL1 and expression of CHL1 in different tissues.

- (a) Western blot analysis of brain derived immunopurified L1 (lane 1 (2  $\mu$ g)), N-CAM (lane 2 (2  $\mu$ g)), MAG (lane 3 (2  $\mu$ g)), and recombinant anion exchange 20 chromatography purified CHL1 protein fragment (lane 4 (0.1  $\mu$ g)) using CHL1 antibodies. (b) Western blot analysis of soluble (S) and insoluble (M) fractions of detergent lysates of crude membranes from brain (lane 1), liver (lane 2), lung (lane 3), kidney (lane 4), and intestine (lane 5) of nine-day-old mice. The numbers at the left (b) refer to the molecular masses of CHL1 immuno-reactive bands of brain 25 (lane 1) and liver (lane 2).

Molecular mass standards are indicated in kD at the left (a) and right (b) margins.

FIGURE 23 shows the detection of CHL1 on transiently transfected COS-1 cells. Monolayer cultures of CHL1-transfected (a) and mock-transfected (c) COS-1 cells were immunostained with polyclonal antibodies against CHL1. (b,d) corresponding phase contrast micrographs for (a,c), respectively. Bar in d = 30  $\mu$ m for a to d.

- 5 FIGURE 24 depicts the localization of CHL1 and L1 mRNA in sections of mouse retina, optic nerve, and cerebellar cortex by *in situ* hybridization analysis. In the retina of 7-day-old mice, L1 mRNA is detectable in ganglion cells located in the ganglion cell layer (1 in a) and in amacrine and horizontal cells located in the inner nuclear layer (2 in 1). Other cells types in the retina or glial cells in the optic
- 10 nerve do not contain detectable levels of L1 transcripts (a). CHL1 mRNA is weakly detectable in ganglion cells and in a few cells located at the inner (i.e. vitread) margin of the inner nuclear layer (b). Glial cells located in proximal (i.e. retina-near) regions of the optic nerve are strongly labeled by the CHL1 antisense cRNA probe whereas glia cells located in more distal regions are only weakly labeled (b).
- 15 In the cerebellar cortex of two-week-old mice, L1 transcripts are detectable in stellate and basket cells in the molecular layer (mol) and in Golgi and granule cells in the internal granular layer (Igl:d). CHL1 transcripts are distributed in a similar pattern, with the only exception that hardly any labeling is visible in thinner part of the molecular layer (b). Sections hybridized with a CHL1 sense cRNA probe are
- 20 not labeled (for a 7-day-old retina and optic nerve, see c).  
Bar in c = 100  $\mu$ m for a-c; bar in e = 150  $\mu$ mm for d and e.

FIGURE 25 illustrates the immunofluorescence microscopic localization of CHL1 in cultures of astrocytes.

- Double-immunolabeling of cultured mouse astrocytes was performed with
- 25 polyclonal antibodies to CHL1 (a,d) and monoclonal antibodies to GFAP (b,e), (c and f) are the corresponding phase contrast micrographs for (a,b and d,e), respectively. Bars in c and F = 20  $\mu$ m for a-c and d-f respectively.

FIGURE 26 is a Western blot analysis of deglycosylated CHL1

Soluble (S) and insoluble (M) fractions of detergent lysates of crude membranes from brain of seven-day-old mice were incubated with N-glycosidase F (N). O-glycosidase (O), both enzymes (N+O), or without enzyme (-) and reacted with antibodies against CHL1 in Western blots. Molecular mass standards are indicated  
5 in kD at the right margin. The molecular masses of the glycosylated and deglycosylated CHL1 protein components are indicated in kD in the box below.

FIGURE 27 shows the presence of the MNK-1 carbohydrate in CHL1 immunoprecipitates from brain tissue. CHL1 was immunoprecipitated from detergent lysates of whole brain tissue of nine-day-old mouse brain using CHL1  
10 antibodies. Brain lysate (lane 1) and immunoprecipitates (lanes 2,3) were resolved by SDS-PAGE, blotted, and incubated with monoclonal antibody 312 against the HNK-1 epitope (lanes 1,2) or CHL1 antibodies (lane 3). Molecular mass standards are indicated in kD at the right margin.

#### FIGURE 28: NEURITE OUTGROWTH OF HIPPOCAMPAL NEURONS IN 15 COCULTURES WITH L929-TRANSFECTANTS

Hippocampal neurons derived from rats of embryonal day 18 were cultured in subconfluent monolayers of L929-transfectants or parental L929 cells. After 11-12 h of coculture the cells were fixed and labeled with monoclonal antibody 412 (recognizing the HNK-1 carbohydrate epitope) or a polyclonal antibody against  
20 NCAM. For measurement of the total neurite length only the longest neurite per each branch was determined due to the highly branched character of the neurons in these cultures.

(A) Neurite outgrowth is promoted by CHL1 and inhibitable by antibodies. Neurons were cocultured with CHL1-transfectants (CHL1) or parental L929 cells  
25 (L929) with (+AB) or without polyclonal antibodies against recombinant CHL1 (500 µg/ml of purified IgG, added 45 min after plating) and on L1-transfectants. The mean total neurite length of 4 - 5 independent experiments is shown. Error bars are standard error-of-the-mean.

- (B) Different CHL1 lines promote neurite outgrowth better than L1. Neurons were cultured on two different CHL1-transfectants (CHL1 line 1, CHL1 line 2) with slightly different expression levels, parental L929 cells (L929) and L1 transfectants (L1). Total neurite length is given as percent of L929 cells as a control (ctr). Error bars are standard error of the mean.
- (C) Neurite outgrowth promotion affects all length classes of neurites. Cumulative frequency distribution plot of the total neurite length of hippocampal neurons cocultured with CHL1-transfectants (CHL1 line 1 and 2) and parental L929 cells (L929) with (+AB) or without antibody treatment as given in (A). The percentage of neurons with neurites longer than or equal to a certain length  $x$  (vertical axis) was plotted as a function of neurite length  $x$  (horizontal axis). Values are from one representative experiment.

FIGURE 29: NEURITE OUTGROWTH OF SMALL CEREBELLAR NEURONS IN COCULTURE WITH L929-TRANSFECTANTS

- Cerebellar neurons derived from 6-7 day old mice were cultured for 20 h on CHL1-transfectants (CHL1), CHL1-transfected non-expressing L929 cells (Mock), parental L929, or L1-transfectants (L1). The staining of the cells was performed as already described in Figure 7.
- (A) CHL1 promotes neurite outgrowth of small cerebellar neurons. The mean of total neurite length of three experiments is shown. Error bars are standard error of the mean.
- (B) CHL1 promotes neurite outgrowth also of small cerebellar neurons better than L1. The total neurite length is given as percent of L929 cells as a control (ctr). Error bars are standard error of the mean.
- (C) Increase of neurite outgrowth of cerebellar neurons by CHL1 affects all size classes of neurites. Cumulative frequency of distribution plot of the total neurite length of the percentage of neurons with neurites longer than or equal to a certain length  $x$  (vertical axis) was plotted as a function of neurite length  $x$  (horizontal axis). Values from one representative experiment are shown.

FIGURE 30: NEURITE OUTGROWTH OF HIPPOCAMPAL NEURONS  
TREATED WITH SOLUBLE CHL1

Hippocampal neurons were cultured on poly-L-lysine coated coverslips for 12 h with addition of supernatants (40 µg/ml of total protein) of crude membrane preparations of CHL1-transfectants (CHL1), parental L929 cells (L929), or L1-transfectants (L1). Staining and measurement of neurite length was performed as already described (Figure 7).

(A) Soluble CHL1 from L929 transfectants promotes outgrowth of the longest and the sum of all neurites per cell. Absolute length of longest neurite and total neurite length are shown. Values are means of three independent experiments. Error bars are standard error of the mean.

(B) Soluble CHL1 promotes a slight increase of neurite number. Total neurite length in percent of the neurite length of hippocampal neurons treated with supernatants derived from parental L929 cells (ctr) are plotted. Values are means of three independent experiments. Error bars are standard error of the mean.

(C) Also soluble CHL1 affects neurite outgrowths of all length classes of neurites. Cumulative frequency distribution plots of the total neurite length from one representative experiment are shown.

FIGURE 31: QUANTITATIVE AGGREGATION ANALYSIS AND STABILITY  
OF CHL1- AND L1-PROTEIN IN L929 TRANSFECTANTS.

(A) Quantitative analysis of aggregation of S2 cell transfectants. To detect aggregation CHL1- (CHL1) (ctr) and L1- (L1) transfected cells were cultured (at densities of about  $3 \times 10^6$  cells/ml) for 18 h in culture medium with (+ind) or without (-ind) induction of transgene expression by  $\text{CuSO}_4$ . Particle number was counted in a hemacytometer at the beginning and at the end of the incubation. The percentage of aggregation was calculated by the index  $(1 - N/\text{NO}) \times 100$ . N18 and NO represent the particle numbers at the end or the beginning of the incubation period, respectively. Values are the means of at least four independent experiments. Error bars are standard deviations.



- (B) Kinetics of aggregation of L929-transfectants. CHL1-transfected (CHL1), CHL1-transfected non-expressing (Mock), parental L929 (L929), and L1-transfected (L1) cells had been detached from tissue culture by treatment with low concentration of trypsin-EDTA, washed and incubated at 37°C in polystyrene tubes.
- 5 An aliquot of each sample was withdrawn every 30 min and the particle number was counted in a hemacytometer. The results are expressed as described in (A). Values shown are the means of at least three independent experiments. Bars are standard deviations.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

#### OF THE INVENTION

- 10 More particularly, the present invention relates to the use of certain agents identified herein as "CNS neural growth modulators" (CNGMs), and particularly to a class of neural cell adhesion molecules as defined herein, to promote neurite outgrowth in the central nervous system (CNS). In general, neurons in the adult
- 15 central nervous system have been considered incapable of regrowth, due to inhibitory molecular cues present on glial cells. The agents and methods of the present invention can be used to overcome this inhibition and promote CNS neurite outgrowth.

- The agents of the invention include and may be selected from any cell adhesion
- 20 molecule which is capable of modulating or promoting CNS neurite outgrowth, and particularly to molecules belonging to the immunoglobulin superfamily. More particularly, the molecules are selected from the members of the immunoglobulin superfamily which mediate  $\text{Ca}^{2+}$ -independent neuronal cell adhesion, including L1, N-CAM and myelin-associated glycoprotein. The invention also contemplates
- 25 fragments of these molecules, and analogs, cognates, congeners and mimics of these molecules which have neurite-promoting activity. Particularly preferable structural motifs for these fragments and analogs include domains similar to the fibronectin type III homologous repeats (particularly repeats 1-2) and immunoglobulin-like domains (particularly domains I-II, III-IV and V-VI).

As the agents of the invention, and particularly, the members of the L1 CAM family, exhibit homophilic binding, both the agents and their antagonists, and particularly, their antibodies, may serve as agonists with respect to the receptor for the agents, and may thus be employed in both diagnostic and therapeutic  
5 applications in the same manner and for the same purpose as the agents themselves. Thus, L1 acts as a receptor, and its antibody may be employed as an agonist, to promote neurite outgrowth as set forth herein, to assist in neural regeneration particularly in the CNS. This capability is further demonstrated in the ability of  
10 the antibodies to L1 to serve in a method for the identification of further members of the L1 CAM family of neural recognition molecules, that will serve as agents herein, and the invention accordingly extends to the molecules that are identified, isolated and characterized by means of such antibodies. As such, therefore, the class of materials identified as CNS neural growth modulators hereinbelow, is  
15 considered to include the antibodies to CAMs such as L1 and its analogs, such as CHL1, described later on herein, among its numbers.

The present invention relates in one aspect to the ectopic expression of CNS neural growth modulators (CNGMs) or neural cell adhesion molecules on differentiated astrocytes *in vivo*. These molecules have been found to enhance neurite outgrowth on monolayer cultures of such astrocytes and cryostat sections of unlesioned and  
20 lesioned adult mouse optic nerves, and also *in vivo*, in optic nerve crush experiments in transgenic animals. The increased neurite outgrowth-promoting capacity is proportional to the level of ectopic CNGM expression. This is demonstrated by comparisons of the distinct transgenic lines of the invention, which express different basal levels of transgenic-encoded CNGM, and by  
25 correlations following increased CNGM expression after a lesion of the optic nerve.

It should be appreciated that although optic nerves, both lesioned and unlesioned, are suitable for use with the present invention, that any part of the nervous system can likewise be used, including portions of the brain and spinal cord.

Neurite outgrowth is dependent on the levels of CNGM expression by astrocytes, demonstrating the specific effect exerted by CNGM in promoting neurite outgrowth in the transgenic animal. Inhibition of neurite outgrowth by polyclonal CNGM antibodies, but not by antibodies to mouse liver membranes, further supports this  
5 specificity, in particular, since both antibodies react well with the cell surfaces of neurons and astrocytes of transgenic animals.

In a preferred embodiment, the CNGM is L1. L1's biological effects can be inhibited by L1 antibodies, which indicates that L1 is homophilically active in a trans configuration at the cell surface of transgenic astrocytes. Furthermore, L1  
10 species-specific antibodies that do not react with chicken dorsal root ganglion neurons inhibit neurite outgrowth of this neuronal cell type on transgenic astrocytes. These findings unequivocally identify L1 as a trans-acting active molecule and show that ectopic expression of L1 by glial cells that normally lack L1 expression significantly enhances neurite outgrowth *in vitro*.

15 The transgene-mediated enhancement of neurite outgrowth on glial cells that do not normally express L1 *in vivo* indicates that glial cells of the adult mammalian central nervous system can be made more conducive to neurite outgrowth. The loss of neurite outgrowth-promoting glia-derived molecules with maturation (Smith et al. (1986) *J. Comp. Neurol.* 251:23-43; Smith et al. (1990) *Dev. Biol.* 138:377-  
20 390) therefore appears to be compensated for by expression of a recognition molecule that is normally highly expressed by glial cells in the adult mammalian peripheral nervous system (Niecke et al. (1985); Bixby et al. (1988) *J. Cell. Biol.* 107:353-362; Seilheimer et al. (1988) *J. Cell. Biol.* 107:341-351).

The phenotype of adult astrocytes from the present transgenic lines may be  
25 modified towards the more Schwann cell-related capacity of reexpressing L1 after infliction of a lesion. An increase in L1 expression by Schwann cells is likely mediated by neurotrophins upregulated after damage by autocrine mechanisms (Seilheimer et al. (1987) *EMBO J.* 6:1611-1616). Similarly, L1 expression by

astrocytes in culture can be upregulated by TGF- $\beta$  and NGF (Saad et al. (1991)). By generating mice with a GFAP-L1 transgene, the inability of mature astrocytes to respond to neural injury is overcome with an upregulation of the neurite outgrowth promoting molecule L1. The expression of L1 may be particularly beneficial for  
5 neurite outgrowth in myelinated tracts of the central nervous system which normally contain several molecules that are neurite outgrowth inhibiting (Schachner et al., *Perspectives in Developm. Neurobiol. in Press*; Schwab et al. (1995) *Ann. Rev. Neurosci.* 16:565-595).

The present invention demonstrates that the inhibitory action of astroglial and  
10 oligodendroglial cells may be overcome, at least in part, by the neurite outgrowth promoting properties of the agents defined herein, and as particularly illustrated by the activity of ectopically expressed L1. Expression of L1 by astrocytes seems also to compensate for inhibitory effects exerted by oligodendrocytes. Permissive and non-permissive molecular cues therefore may not have to be localized on the same  
15 cell type for neurite outgrowth to occur. Instead, such molecular cues might be partitioned among different cell types. The cellular and molecular manipulation of L1 and other neurite outgrowth promoting molecules may therefore allow enhancement of the regenerative capacity of the adult mammalian central nervous system following injury or disease.

20 As indicated earlier, the present invention extends to the promotion of neural growth in the CNS, including such growth as is desired to regenerate structures lost due to injury or illness, as well as those structures and tissues exhibiting incomplete or immature formation. The agents of the invention also exhibit a neuroprotective or neuropreservative effect as illustrated later on herein, and for example, could be  
25 administered to inhibit or counteract neural degeneration or loss of variable etiology.

The invention accordingly extends to constructs and compositions containing or delivering the agents of present invention, whether by the promotion of the

expression of certain agents via gene therapy or the like, or by the exogenous administration of the agents where appropriate and beneficial, in pharmaceutical compositions to treat injured or diseased CNS structures. In this latter connection, it is contemplated that certain of the agents are able to exert a growth promoting effect when so administered, although it is recognized that members of the presently identified group, such as L1 and N-CAM appear to bind homophilically and may therefore prove more beneficial when delivered by means of expression. The invention is intended to extend to both routes and protocols where feasible.

It should also be appreciated that the present invention relates to the use of CNGM-secreting cells for the modulation of neural outgrowth, regeneration, and neural survival in the CNS. As such, certain soluble CNGMs and fragments thereof, and cognate molecules thereof are also within the invention.

Therefore, if appearing herein, the following terms shall have the definitions set out below.

The terms "agent", "CNS neural growth modulator", "CNGM", "neural recognition molecule", "recognition factor", "recognition factor protein(s)", "neural adhesion molecule", and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and extends to those proteins having the amino acid sequence previously described and the profile of activities set forth herein and in the Claims. The foregoing terms also include active fragments of such proteins, cognates, congeners, mimics and analogs, including small molecules that behave similarly to said agents.

Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex

or its named subunits. Also, the terms "CNS neural growth modulator", "CNGM", "neural recognition factor", "recognition factor", "recognition factor protein(s)", and "neural adhesion molecule" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is retained by the polypeptide. NH<sub>2</sub> refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J. Biol. Chem.*, 243:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

| TABLE OF CORRESPONDENCE |                 |                 |               |
|-------------------------|-----------------|-----------------|---------------|
| SYMBOL                  |                 | AMINO ACID      |               |
|                         | <u>1-Letter</u> | <u>3-Letter</u> |               |
|                         | Y               | Tyr             | tyrosine      |
|                         | G               | Gly             | glycine       |
| 20                      | F               | Phe             | phenylalanine |
|                         | M               | Met             | methionine    |
|                         | A               | Ala             | alanine       |
|                         | S               | Ser             | serine        |
|                         | I               | Ile             | isoleucine    |
| 25                      | L               | Leu             | leucine       |
|                         | T               | Thr             | threonine     |
|                         | V               | Val             | valine        |
|                         | P               | Pro             | proline       |
|                         | K               | Lys             | lysine        |
| 30                      | H               | His             | histidine     |

|   |   |     |               |
|---|---|-----|---------------|
|   | Q | Gln | glutamine     |
|   | E | Glu | glutamic acid |
|   | W | Trp | tryptophan    |
|   | R | Arg | arginine      |
| 5 | D | Asp | aspartic acid |
|   | N | Asn | asparagine    |
|   | C | Cys | cysteine      |

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

15 A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described

herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

5 An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

10 A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

15 Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

20 A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site  
25 (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus-sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT"



boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is  
5 "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the  
10 host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

The term "oligonucleotide", as used herein in referring to probes, is defined as a  
15 molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is  
20 capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently  
25 long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for

diagnostic applications. depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides. although it may contain fewer nucleotides.

5 The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to  
10 the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

As used herein, the terms "restriction endonucleases" and "restriction enzymes"  
15 refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of  
20 the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of  
25 the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

- Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.
- 10 A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a
- 15 heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.
- 20 An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567.
- 25 An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially  
5 intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F(ab')<sub>2</sub> and F(v), which portions are preferred for use in the therapeutic methods described herein.

Fab and F(ab')<sub>2</sub> portions of antibody molecules are prepared by the proteolytic  
10 reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from F(ab')<sub>2</sub> portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and  
15 followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of  
20 immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

25 The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an

allergic or similar untoward reaction. such as gastric upset, dizziness and the like, when administered to a human.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 30 percent, more preferably by at least 50 percent, most preferably by at least 90 percent, a clinically significant change in the S phase activity of a target cellular mass, or other feature of pathology such as for example, elevated blood pressure, fever or white cell count as may attend its presence and activity.

A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5x SSC and 65°C for both hybridization and wash.

In one aspect, the present invention relates to transgenic animals which express a CNGM or neural recognition molecule, in particular L1, and preferably in astrocytes. These animals have increased capability for neural outgrowth in the central nervous system.

The invention also includes an assay system for the screening of potential drugs effective to modulate neural outgrowth of target mammalian cells by interrupting or

potentiating the CNGM's neural recognition activity. By "neural recognition activity" or "neural adhesion activity" is meant any biological effect which is a result of the CNGM's binding to another molecule, including intracellular effects on second messengers. In one instance, the test drug could be administered either  
5 to a cellular sample with the ligand that activates the CNS neural growth modulator, or a transgenic animal expressing the CNS neural growth modulator, to determine its effect upon the binding activity of the modulator to any chemical sample, or to the test drug, by comparison with a control. Identifying characteristics of at least one of the present CNS neural growth modulators, in  
10 particular L1, is its participation in changes in steady state levels of intracellular messengers, including  $\text{Ca}^{2+}$ , pH, and cyclic nucleotides, as well as changes in the activities of protein kinases such as protein kinase C, pp60<sup>c-src</sup>, a casein type II kinase and another kinase known to phosphorylate L1.

The assay system could more importantly be adapted to identify drugs or other  
15 entities that are capable of binding to the CNGMs or proteins, either in the cytoplasm or in the nucleus, thereby inhibiting or potentiating transcriptional activity. Such assay would be useful in the development of drugs that would be specific to particular cellular activity, such as neural outgrowth or increase in synaptic efficacy, or that would potentiate such activity, in time or in level of  
20 activity. For example, such drugs might be used to modulate neural outgrowth in response to injury, or to treat other pathologies, as for example, in treating neurodegenerative diseases such as Parkinson's Disease, ALS, Huntington's Disease and Alzheimer's Disease.

In yet a further embodiment, the invention contemplates agonists and antagonists of  
25 the activity of a CNS neural growth modulator. In particular, an agent or molecule that inhibits the ability of neurons to recognize a CNGM such as L1 can be used to block neural outgrowth, where such outgrowth is contraindicated, and as described earlier, a pharmaceutical composition containing such an agent may be administered directly to the target site. In another embodiment, an agonist can be a peptide

having the sequence of a portion of an L1 domain particularly that between fibronectin type III homologous repeats 2 and 3, or an antibody to that region. Either of these molecules may potentially be used where a particular CNGM such as L1 has the ability to undergo homophilic binding (i.e., L1 can bind to itself, and  
5 therefore both antibodies to L1 and fragments of L1 itself are capable of binding to L1).

One of the diagnostic utilities of the present invention extends to the use of the present CNGMs in assays to screen for protein kinase inhibitors. Because the activity of the CNGMs described herein are phosphorylated, they can and  
10 presumably are dephosphorylated by specific phosphatases. Blocking of the specific kinase or phosphatase is therefore an avenue of pharmacological intervention that would modulate the activity of these neural recognition proteins.

The present invention likewise extends to the development of antibodies against the  
15 CNGMs, including naturally raised and recombinantly prepared antibodies. For example, the antibodies could be used to screen expression libraries to obtain the gene or genes that encode the CNGMs. Such antibodies could include both polyclonal and monoclonal antibodies prepared by known genetic techniques, as well as bi-specific (chimeric) antibodies, and antibodies including other  
20 functionalities suiting them for additional diagnostic use conjunctive with their capability of modulating neural outgrowth.

In particular, antibodies against CNS neural growth modulators can be selected and are included within the scope of the present invention for their particular ability in binding to the protein. Thus, activity of the neural growth modulators or of the  
25 specific polypeptides believed to be causally connected thereto may therefore be followed directly by the assay techniques discussed later on, through the use of an appropriately labeled quantity of the neural growth modulator or antibodies or analogs thereof.

Thus, the CNGMs, their analogs, and any antagonists or antibodies that may be raised thereto, are capable of use in connection with various diagnostic techniques, including immunoassays, such as a radioimmunoassay, using for example, an antibody to the CNGM that has been labeled by either radioactive addition,  
5 reduction with sodium borohydride, or radioiodination.

In an immunoassay, a control quantity of the antagonists or antibodies thereto, or the like may be prepared and labeled with an enzyme, a specific binding partner and/or a radioactive element, and may then be introduced into a cellular sample. After the labeled material or its binding partner(s) has had an opportunity to react  
10 with sites within the sample, the resulting mass may be examined by known techniques, which may vary with the nature of the label attached. For example, antibodies against the CNGMs may be selected and appropriately employed in the exemplary assay protocol, for the purpose of following protein material as described above.

15 In the instance where a radioactive label, such as the isotopes  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{36}\text{Cl}$ ,  $^{51}\text{Cr}$ ,  $^{57}\text{Co}$ ,  $^{58}\text{Co}$ ,  $^{59}\text{Fe}$ ,  $^{90}\text{Y}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ , and  $^{186}\text{Re}$  are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric  
20 techniques known in the art.

The present invention includes an assay system which may be prepared in the form of a test kit for the quantitative analysis of the extent of the presence of the neural growth modulators, or to identify drugs or other agents that may mimic or block their activity. The system or test kit may comprise a labeled component prepared  
25 by one of the radioactive and/or enzymatic techniques discussed herein, coupling a label to the neural growth modulators, their agonists and/or antagonists, and one or more additional immunochemical reagents, at least one of which is a free or immobilized ligand, capable either of binding with the labeled component, its



binding partner. one of the components to be determined or their binding partner(s).

In a further embodiment, the present invention relates to certain therapeutic methods which would be based upon the activity of the CNS neural growth modulator(s), its (or their) subunits, or active fragments thereof, or upon agents or other drugs determined to possess the same activity. A first therapeutic method is associated with the promotion of CNS neural growth resulting from the presence and activity of the CNGM, its active fragments, analogs, cognates, congeners or mimics, and comprises administering an agent capable of modulating the production and/or activity of the CNGM, in an amount effective to promote CNS development, regrowth or rehabilitation in the host. Conversely, drugs or other neutralizing binding partners to the CNGM or proteins may be administered to inhibit or prevent undesired neural outgrowth. Also, the modulation of the action of specific kinases and phosphatases involved in the phosphorylation and dephosphorylation of CNGMs or proteins presents a method for modulating the activity of the modulator or protein that would concomitantly potentiate therapies based on CNGM/protein activation.

More specifically, the therapeutic method generally referred to herein could include the method for the treatment of various pathologies or other cellular dysfunctions and derangements by the administration of pharmaceutical compositions that may comprise effective inhibitors or enhancers of the activity of the CNS neural growth modulator or its subunits, or other equally effective drugs developed for instance by a drug screening assay prepared and used in accordance with a further aspect of the present invention. For example, drugs or other binding partners to the CNS neural growth modulator or proteins may be administered to inhibit or potentiate binding and second messenger activity.

As mentioned above, the invention extends to the discovery of a full family of L1 CAMs, and particularly to an analog to L1 known as CHL1. CHL1 comprises an

N-terminal signal sequence, six immunoglobulin (Ig)-like domains, and 4.5 fibronectin type III (FN)-like repeats, a transmembrane domain, and a C-terminal, most likely intracellular domain of approximately 100 amino acids. CHL1 is most similar in its extracellular domain to chicken Ng-CAM (about 40% amino acid identity), followed by mouse L1, chicken neurofascin, chicken Nr-CAM, *Drosophila* neuroglian, and zebrafish L1.1 (37 to 28 % amino acid identity, respectively), and mouse F3, rat TAG-1, and rat BIG-1 (about 27% amino acid identity). The similarity with other members of the Ig superfamily (e.g. N-CAM, DCC, HLAR, rse) is 16 to 11 %. The intracellular domain is most similar to mouse and chicken Nr-CAM, mouse and rat neurofascin (about 50 % amino acid identity) followed by chicken neurofascin and Ng-CAM, *Drosophila* neuroglian, and zebrafish L1.1 and L1.2 (about 40 % amino acid identity). Besides the high overall homology and conserved modular structure among previously recognized members of the L1 family (mouse/human L1/rat NILE; chicken Ng-CAM; chicken/mouse Nr-CAM; *Drosophila* neuroglian; zebrafish L1.1 and L1.2; chicken/mouse neurofascin/rat ADGP), L1 characteristic criteria were identified with regard to the number of amino acids between positions of conserved amino acid residues defining distances within and between two adjacent Ig-like domains and FN-like repeats. These show a colinearity in the six Ig-like domains and adjacent four FN-like repeats that is remarkably conserved between L1 and molecules containing these modules (designated the L1 family cassette) including the GPI linked forms of the F3 subgroup (mouse F3/chicken F11/human CNTN1; rat BIG-1/mouse PANG; rat TAG-1/mouse TAX-1/chicken axonin-1). The colorectal cancer molecule (DCC) previously introduced as an N-CAM like molecule conforms to the L1 family cassette. Other structural features of CHL1 shared between members of the L1 family are a high degree of N-glucosidically linked carbohydrates (about 20% of its molecular mass), which include the HNK-1 carbohydrate structure, and a pattern of protein fragments comprising a major 185 kD band and smaller fragments of 100 and 125 kD. As for the outer L1 family members, predominant expression of CHL1 is observed in the nervous system and at later developmental stages.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

### EXAMPLE 1

#### 5 **GFAP-L1 transgene and production of transgenic mice**

Glial fibrillary acidic protein (GFAP, Eng et al. (1971) *Brain Res.* 28:351-354) is expressed predominately by astrocytes at late stages in the development of the mouse central nervous system (Landry et al. (1990) *J. Neurosci. Res.* 25:194-203). Therefore regulatory sequences of the GFAP gene were used to direct the  
10 expression of the neural cell adhesion molecule L1 to mature astrocytes of transgenic mice. The GFAP-L1 transgene (Fig. 1) encodes only the neural cell adhesion molecule L1 since the ATG of the GFAP gene was mutated and the L1 coding sequence is followed 3' by a translational stop and a polyadenylation signal (Toggas et al. (1994) *Nature* 367:188-193). This construct was used to establish  
15 three different lines of transgenic mice, designated 3418, 3426 and 3427.

The mouse L1 cDNA (Moos et al. (1988) *Nature* 334:701-703) was inserted into exon 1 of the murine glial fibrillary acidic protein (GFAP) gene modified as described previously (Toggas et al. (1994) *Nature* 367:188-193). The 4.05 kb mouse L1 cDNA containing the entire coding sequence of the protein and 250 3'  
20 non-translated nucleotides was fused with the modified GFAP-L1 transgene.

The 14.5 kb GFAP-L1 transgene was excised from a modified cloning vector by digestion with *Sfi* I, followed by electrophoresis and electroelution from an agarose gel. Purified DNA was diluted to a final concentration of 2 µg/ml in T<sub>3</sub>E<sub>0.1</sub> (5 mM Tris-HCl, pH 7.4, 0.1 mM EDTA). Approximately 2 µl of diluted DNA were  
25 microinjected into the male pronucleus of fertilized eggs derived from CB6F1 females (superovulated) mated to C57B1/6J males. Eggs surviving the micromanipulation were transferred into oviducts of pseudo-pregnant foster mothers

following describes methods (Hogan et al. (1986) *Manipulating Mouse Embryo*, Cold Springs Harbor Laboratory, New York).

## EXAMPLE 2

### **Southern blot analysis**

- 5 Mice were analyzed for the integration of the transgene into the mouse genome by Southern blot analysis of genomic DNA isolated from tail biopsies (Southern (1975) *J. Mol. Biol.* 98:503-517). Transgenic founder mice were mated and pups screened in the same manner to establish transgenic lines. Ten  $\mu$ g samples of DNA were digested with either *Bam* HI or with *Eco* RI and *Xba* I followed by
- 10 electrophoretic separation on a 0.7% agarose gel and transfer to Hybond N+ membrane (Amersham) under alkaline conditions. A 3.3 kb *Eco* RI-fragment of the L1 cDNA or a 330 bp *Hind* III fragment of SV40 late splice and polyadenylation site purified from A1.5 plasmid (Maxwell et al. (1989) *Biotechniques* 7:276-280) were labelled with  $^{32}\pi\alpha$ -CTP by random priming
- 15 (Boehringer Mannheim) for use as probes. Prehybridization was performed at 65°C for one hour in 5x SSPE, 5x Denhardt's solution, 0.5% (w/v) SDS and 0.1 mg/ml sonicated non-homologous DNA. Hybridization was performed overnight. Final stringency wash conditions for all Southern blots were 0.1x SSPE and 0.1% SDS (w/v) at 65°C.

20

## EXAMPLE 3

### **Northern blot analysis**

- Anaesthetized adult mice (12-weeks-old) were sacrificed by a lethal dose of chloralhydrate and brains were removed and immediately frozen in liquid nitrogen. Total cellular RNA was isolated by pulverizing the tissue in liquid nitrogen. Four
- 25 molar guanidinium thiocyanate was added to the pulverized tissue. Isolation of total RNA was performed as described (Chomczynski et al. (1987) *Anal. Biochem.* 162:156-159; Pagliusi et al. (1989) *AMOG. J. Neurosci. Res.* 22:113-119). RNA yields were estimated from absorbance at 260 nm. Ten  $\mu$ g of the RNA were

fractionated on 1% agarose-formaldehyde gels for Northern blot analysis (Thomas (1980) *Proc. Natl. Acad. Sci. USA* 77:201-205).

Randomly primed L1 cDNA probes were used to simultaneously detect the endogenous L1 mRNA of 6 kb (Tacke et al. (1987) *Neurosci. Lett.* 82:89-94) and  
5 the transgene-derived L1 mRNA of 4.2 kb. Densitometric analysis of Northern blots was performed on scanned images (Arcus scanner, Agfa-Gavaert) of the original films using the Image Program (NIH, Research Services Branch, NIMH).

Northern blot analysis of total RNA from whole brains of the transgenic animals revealed L1 transcripts of a size (4.2 kb) expected for transgene-derived mRNA  
10 (Fig. 2.). These transcripts are clearly distinct from the endogenous L1 mRNA which is 6 kb and derived from postmitotic neurons. Densitometric analysis revealed that the levels of transgene-derived L1 mRNA were 34%, 13% and 8% in lines 3426, 3427 and 3418, respectively, as compared to the levels of endogenous L1 mRNA (rated 100%).

15

#### EXAMPLE 4

##### **Animals**

For cultures on cryostat sections, immunocytochemistry and *in situ* hybridization experiments, control animals were taken from stocks of age-matched normal C57b1/6J mice or non-transgenic littermates. For isolation of small cerebellar  
20 neurons and for preparation of astrocyte cultures six-day-old ICR non-transgenic pups were used. Dorsal root ganglion (DRG) neurons were prepared from eight-day-old chick embryos.

**EXAMPLE 5*****In situ* hybridization**

To verify that astrocytes of transgenic animals expressed L1 *in vivo*, optic nerves were analyzed by *in situ* hybridization. The optic nerve was chosen since it  
5 contains only glial cells and is free of neuronal cell bodies. Astrocytes *in vivo* normally lack expression of L1 at any developmental stage (unpublished data).

For detection of L1 mRNA in cryostat sections of fresh-frozen brain sections, digoxigenin-labelled cRNA was generated by *in vitro* transcription (Dörries et al. (1993) *Histochemistry* 99:251-262). The sequence encoding the extracellular part  
10 of L1 (Moos et al. (1988) was subcloned into the pBluescript KS+ (Stratagene) vector. Anti-sense and sense cRNA probes were generated by transcribing the L1 insert after linearization of the resulting plasmid with *Xho* I or *Xba* I, using the T7 and T3 promoters, respectively. For generation of GFAP cRNA probes, a 1.2 kb fragment of GFAP cDNA (Lewis et al. (1984) *Proc. Natl. Acad. Sci.* 81:2743-  
15 2745; kindly provided by Dr. N.J. Cowan) encoding the N-terminus of the protein was subcloned into the pBluescript KS+ vector. Anti-sense and sense cRNA probes were generated by transcribing the resulting plasmid, linearized with *Eco* RI and *Xho* I, from the T3 and T7 promoters, respectively. To improve tissue penetration, anti-sense and sense probes were sized under alkaline hydrolysis  
20 conditions to obtain an average fragment length of about 300 nucleotides. *In situ* hybridization on sections of optic nerves prepared from adult (12-weeks-old) animals was performed as described by elsewhere (Dörries et al. (1993); Bartsch et al., *J. Neurosci.*, in press).

In non-transgenic controls L1 transcripts were detected only in nerve cells of the  
25 retina but not in optic nerve, neither before (Fig. 3A) nor after a lesion (Fig. 3B). By contrast, L1 mRNA was expressed by glial cells of the optic nerves from transgenic mice (Fig. 3C). L1 mRNA positive cells were detectable in both the distal myelinated and the proximal-unmyelinated parts of the nerve. The intensity

of the hybridization signal was higher in the unmyelinated proximal part, when compared to the myelinated distal part of the nerve.

A similar distribution of positive cells and similar differences in labelling intensity between unmyelinated and myelinated regions were observed using a GFAP cRNA probe (compare Figs. 3C and E). The number of L1 mRNA positive cells in the optic nerve of transgenic animals was, however, always significantly lower than the number of GFAP-positive cells, probably due to the lower sensitivity of the L1 cRNA probe. Alternatively, detectable levels of L1 mRNA might be achieved only in astrocytes with high levels of GFAP expression. Such a threshold effect could be due to the design of the GFAP-L1 transgene which contains only 2 kb of GFAP 5' flanking sequences. *In vitro* studies suggest that the region between 2 and 6 kb upstream of the transcriptional start site contains sequence elements augmenting expression of GFAP-driven fusion genes in C6 cells (Sarid (1991) *J. Neurosci.* 28:217-228). Finally, the modification of the GFAP exon 1, including the introduction of the large L1 cDNA, might reduce the stability of the chimeric mRNA as compared to GFAP mRNA and alter effects exerted by regulatory GFAP sequences located upstream and downstream of the modified region.

After lesioning the optic nerve, an upregulation of L1 expression was observed in transgenic (Fig. 3D) but not in nontransgenic (Fig. 3B) optic nerves. The number of cells which expressed L1 and the intensity of the L1 hybridization signal were similar in different individuals of the same transgenic line but varied across different transgenic lines. Consistent with the results obtained by Northern blot analysis (see above). L1 mRNA positive cells were most abundant in line 3426 followed by line 3427 and, finally, line 3418. This variability in the level of transgene expression in different lines could be related to a number of factors, in particular, effects caused by the neighboring host chromatin regions flanking the different transgene integration sites (Proudfoot (1986) *Nature* 322:562-565; Reik et al. (1987) *Nature* 328:248-251; Sapienze et al. (1987) *Nature* 328:251-254).

**EXAMPLE 6****Antibodies**

Production of polyclonal rabbit antibodies against mouse L1 and purification on an L1 immunoaffinity column (Rathjen et al. (1984); Martini et al. (1988) and  
5 polyclonal antibodies against mouse liver membrane (Lindner et al. (1983); Pollerberg et al. (1985) have been described. A mouse monoclonal antibody against GFAP was purchased (Boehringer Mannheim).

For Western blot analysis, polyclonal and monoclonal antibodies were visualized by horseradish peroxidase conjugated goat anti-mouse or rabbit antibodies (Dianova,  
10 Hamburg, Germany). For immunocytochemistry, primary antibodies were detected using fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit and goat anti-mouse antibodies (Dianova). Digoxigenin-labelled cRNA probes for *in situ* hybridization were visualized by alkaline phosphatase-conjugated Fab fragments to digoxigenin (Boehringer Mannheim).

15

**EXAMPLE 7****Maintenance of neurons on cryostat sections**

To analyze whether optic nerves from transgenic animals are more conducive to neurite outgrowth than optic nerves from wild type animals, cerebellar neurons were maintained on cryostat sections of lesioned and contralateral unlesioned optic  
20 nerves (Fig. 7).

Optic nerves of 6 to 16-week-old mice were prepared as described by Bartsch et al. (1989) *J. Comp. Neurol.* 284:451-462. In brief, lesioned and unlesioned optic nerves were embedded and frozen in serum-free, hormonally defined medium (Fischer (1986b) *Neurosci. Lett.* 28:325-329) using liquid nitrogen. Tissue sections  
25 (14  $\mu$ m thick) were cut longitudinally on a Frigocut 270-cryostat (Jung-Reichardt), mounted onto poly-L-lysine-coated (Sigma, 0.001% in water) sterile glass coverslips and air-dried for 2-3 hours in a sterile chamber. After washing the sections for 5 minutes with medium. Percoll gradient-purified small cerebellar



neurons (Keilhauer et al. (1985) *Nature* 316:728-730) from six-day-old ICR mice ( $6 \times 10^4$  cells in 100  $\mu$ l medium) were applied to each coverslip. Cells were maintained in an incubator at 37°C with a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

- 5 Neurite outgrowth was also measured in the presence of antibodies. Sections were pre-incubated with polyclonal L1 antibodies or polyclonal antibodies against mouse liver membranes (100  $\mu$ g/ml, dialyzed extensively against and diluted in culture medium) for 1 hour at 37°C. After removal of antibodies, sections were washed carefully with culture medium (5 times, each for 5 minutes at room temperature)
- 10 and Percoll gradient purified small cerebellar neurons were added. After 2 days, cryostat cultures were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature and the neurite lengths were measured. To avoid "edge effects" in the measurements, we did not evaluate the sections which were situated in the outer rim comprising 20% of the coverslips. Using a semi-automatic computer
- 15 image analysis program (IBAS, Kontron, Zeiss) the lengths of all neurites which had grown on these sections were measured and the average neurite length per neuronal cell body calculated. For each experiment and optic nerve (lesioned or unlesioned), the average length of neurites grown on nerves of transgenic animals was related to the corresponding values of control animals. Twelve independent
- 20 experiments were performed with lesioned and contralateral unlesioned nerves using at least two transgenic animals.

- For transgenic animals, an increase in neurite length was observed on lesioned compared with unlesioned nerves. In contrast, neurite lengths on lesioned and unlesioned optic nerves of wild type animals were not significantly different (Fig.
- 25 8). Neurites of neurons cultured on unlesioned optic nerves from transgenic animals were consistently longer than neurites of neurons cultured on unlesioned nerves from wild type animals. A maximal increase in neurite length of about 300% was observed when using sections from line 3426. Similarly, neurite length

on lesioned nerves of transgenic lines was increased up to 400% when compared with lesioned nerves from wild type animals.

- The neurite outgrowth promoting activity of transgenic optic nerves correlated positively with the level of L1 expression (Fig. 8). Unlesioned optic nerves of line 3426, which express the highest levels of L1 protein were more potent in increasing neurite outgrowth than those of lines 3427 and 3418 expressing, by comparison, lower levels of L1 (in decreasing order). On lesioned optic nerves of lines 3426 and 3427 (28 days after the lesion), neurite outgrowth was four times higher than on lesioned optic nerves of wild type animals. The finding that the increase in neurite outgrowth in lesioned optic nerves was similar for the lines 3426 and 3427 (although line 3426 shows 25% increase in L1 protein expression after lesion as compared with line 3427) could indicate that the level of L1 protein in line 3427 already suffices for maximal induction of neurite outgrowth from small cerebellar neurons.
- Pre-incubation of unlesioned optic nerves from wild type animals with polyclonal antibodies to L1 or mouse liver membranes did not significantly affect neurite lengths (Fig. 9). In contrast, neurite lengths were reduced by more than 50% when cryostat sections of unlesioned or lesioned optic nerves from the transgenic line 3426 were pre-incubated with L1 antibodies (Fig. 9). Antibodies to liver membranes, which strongly bind to optic nerves and small cerebellar neurons (data not shown), did not show similar inhibitory effects. Interestingly, pre-incubation of lesioned optic nerves from wild type animals with L1 antibodies induced an increase in neurite outgrowth compared with lesioned nerves from wild type animals without a prior antibody pre-incubation. Antibodies to mouse liver membranes did not show a significant increase under the same conditions, indicating that addition of cell surface reactive antibodies per se does not disturb neurite outgrowth.

**EXAMPLE 8****Maintenance of neurons on monolayer cultures of astrocytes**

- To prepare astrocyte monolayers, forebrains from six-day-old mice were cleaned free of non-neuronal tissue and dissociated as described elsewhere (Schnitzer et al. (1981) *J. Neuroimmunol.* 1:429-456; Fischer et al. (1982a) *Neurosci. Lett.* 29:297-302; Keilhauer et al. (1985). Cells were maintained on poly-L-lysine-coated (Sigma, 0.001% in water) cell culture flasks in BME medium (Gibco) containing 10% horse serum and 2 mM glutamine for 14 to 21 days. Contaminating oligodendrocytes and neurons were removed by shaking the flasks at every medium change and by subculturing the cells at intervals of four days. Immunostaining for GFAP after 14 days of maintenance showed that more than 90% of the cells were astrocytes. After 14 days in culture, the cells were trypsinized and maintained as monolayers for five days on poly-L-lysine-coated glass coverslips. Percoll gradient purified small cerebellar neurons (Schnitzer et al. (1981)) from six-day-old mice and dorsal root ganglion (DRG) (Seilheimer et al. (1988) *J. Cell. Biol.* 107:341-351) neurons from eight-day-old chick embryos were then added onto the astrocyte monolayers. After 6 hours of co-culture for cerebellar and 12 hours for DRG neurons, the co-cultures were fixed with 2% paraformaldehyde in PBS and neurite lengths were analyzed as described in Example 7.
- Neurite outgrowth from mouse small cerebellar or chick dorsal root ganglion (DRG) neurons was also studied in monolayer cultures of astrocytes derived from transgenic (line 3426) or non-transgenic controls (Fig. 10, Table 1).

**TABLE 1**

**Neurite lengths of cerebellar and dorsal root ganglion (DRG) neurons maintained on astrocytic monolayers prepared from wild type mice (WT) and the transgenic line 3426.**

|                   | Cerebellar<br>neurons | DRG<br>neurons |
|-------------------|-----------------------|----------------|
| WT                | 65 ± 34 mm            | 90 ± 10 mm     |
| WT + anti L1      | 72 ± 30 mm            | 105 ± 14 mm    |
| WT + anti liver   | 57 ± 24 mm            | 107 ± 12 mm    |
| 3426              | 75 ± 41 mm            | 137 ± 10 mm    |
| 3426 + anti L1    | 45 ± 25 mm            | 88 ± 8 mm      |
| 3426 + anti liver | 58 ± 31 mm            | 124 ± 15 mm    |

Neurite lengths on astrocytes without pre-incubation with any antibody or after treatment with polyclonal antibodies against L1 (anti L1) or antibodies against mouse liver membranes (anti liver) are shown. Mean values ± standard deviation are from at least 100 neurons from two independent experiments carried out in quadruplicate.

Neurite length of cerebellar or DRG neurons on transgenic astrocytes was approximately 15% or 50% higher, respectively, when compared with neurite length using wild type astrocytes (Table 1). Anti-liver membrane antibodies did not affect neurite length on astrocyte monolayers from wild type or transgenic animals (Fig. 10, Table 1). Pre-incubation of astrocyte monolayers with L1 antibodies did not significantly affect neurite length on cells from wild type animals. In contrast, it reduced neurite length of cerebellar or DRG neurons grown on cells from transgenic animals by approximately 40%. It is noteworthy in this context that the polyclonal antibodies directed against mouse L1 used in this study do not react with neurons from chicken (Martini et al., 1994a; data not shown). By immunofluorescence analysis it could be shown, however, that these antibodies bind as efficiently as L1 antibodies to astrocytes from transgenic animals as well as to mouse small cerebellar neurons (data not shown).

**EXAMPLE 9****Immunofluorescence and Aurion-GP immunogold microscopy**

L1 and GFAP immunostaining of fresh-frozen cross- or longitudinally sectioned optic nerves or astrocytic monolayers of wild type and transgenic animals were performed as described (Bartsch et al. (1989)). For double-labelling, we first incubated astrocytes as live cells with L1 antibodies (2 µg/ml in 1% BSA in PBS) at 4°C for 30 minutes. After permeabilizing the cells with 70% methanol at -20°C for 10 minutes, cells were incubated with GFAP antibody for 30 minutes at 4°C.

For quantification of neurite lengths in cryostat culture experiments, the Aurion immuno R-Gent silver enhanced staining was used according to the manufacturer's instructions (Aurion, Immuno Gold Reagents & Accessories Custom Labelling, Wageningen, The Netherlands) with minor modifications. In brief, cultures were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature, incubated in 50 mM glycine in PBS for 10 minutes and then treated for 15 minutes in blocking buffer (BB, 0.5% BSA in PBS). After 3 washes in BB, each for 5 minutes, cells were incubated with L1 antibodies diluted in BB (2 µg/ml) for 30 minutes at room temperature. Subsequently, cultures were washed 3 times in BB each for 5 minutes and secondary antibody diluted 1:20 in BB was added for 1 hour at room temperature. After 3 washes with distilled water, cultures were fixed in 2% glutaraldehyde in PBS for 10 minutes at room temperature and washed 3 times with distilled water. A 1:1 mixture of enhancer and developer was then added at room temperature. After the appearance of the reaction product, coverslips were washed 3 times with distilled water and embedded in glycerol.

In optic nerves of non-transgenic mice, L1 immunoreactivity was restricted to unmyelinated retinal ganglion cell axons (Bartsch et al. (1989)). In unlesioned optic nerves from transgenic animals, weak L1 immunoreactivity was also found in association with cell bodies and radially oriented cell processes (Fig. 4A). The intensity of this L1 immunoreactivity in transgenic optic nerves increased significantly after a lesion (Fig. 4B) and was similar in distribution to the GFAP

immunoreactivity found in unlesioned (Fig. 4C) or lesioned (not shown) wild type nerves.

L1 expression was additionally analyzed in cultures of astrocytes prepared from forebrain of six-day-old transgenic animals. No L1 immunoreactivity was  
5 detectable on astrocytes from wild type animals (Fig. 5D). In contrast, L1 positive cells were present in cultures from transgenic animals (Fig. 5A). As demonstrated by double-immunostaining, the same cells also proved positive for GFAP (Fig. 5B and E) indicating that the cells expressing L1 are indeed astrocytes. Since L1  
immunostaining was performed on living cells, it seems likely that in the transgenic  
10 animals L1 is also exposed on the cell surface of astrocytes *in vivo*.

#### **EXAMPLE 10**

##### **Western blot analysis**

To further quantitate the amount of L1 expression in GFAP-L1 transgenic mice, detergent extracts of homogenates of unlesioned and lesioned (15 days after the  
15 lesion) optic nerves from wild type and transgenic adult mice were analyzed on Western blots (Fig. 6).

Lesioned (15 days after the lesion) and contralateral unlesioned optic nerves from 8-week-old animals were cleaned free of non-neuronal tissues and then frozen in liquid nitrogen. Care was taken that only myelinated distal but not L1  
20 immunoreactive unmyelinated or partly myelinated proximal regions of the nerves were used. Nerves were frozen and thawed ten times before sonication with a Branson B15 sonicator at 4°C for 5 minutes. The tissues were then homogenized with a Dounce homogenizer in homogenization buffer (1% Triton X-100, 2 M urea, 5 mM benzamidine, 0.1 mM iodoacetamide, 1 mM phenylmethanesulfonyl  
25 fluoride, 5 mM Na-p-tosyl-L-lysinechloro-methyl ketone in PBS). Homogenates were cleared by centrifugation at 16,000g at 4°C for 15 minutes. Supernatants  
~~were treated with methanol/chloroform to precipitate proteins as described by~~  
Wessel et al. (1984). The protein content was determined in the supernatant

(Pierce). After SDS-PAGE on 7% slab gels under reducing conditions, proteins (25 µg) were analyzed by Western blotting using polyclonal L1 antibodies (0.4 µg/ml). Horseradish peroxidase-conjugated secondary antibody (2 µg/ml) was detected by the ECL Western blotting detection kit (Amersham). Densitometric analysis of immunoblots was performed on scanned images (Arcus scanner, Agfa-Gavaert) of the original films using the Image Program (NIH, Research Services Branch, NIMH).

Densitometric analysis of the immunoblots demonstrated that L1 expression in unlesioned optic nerves of transgenic animals was about 40% and 13% (lines 3426 and 3427, respectively) higher than in unlesioned optic nerves of wild type animals. L1 expression in lesioned transgenic nerves was 310% and 200% (lines 3426 and 3427, respectively) higher as compared with lesioned nerves of wild type animals. A comparison between lesioned and contralateral unlesioned optic nerves from wild type animals revealed a decrease in L1 protein expression of about 40% on the lesioned side. In contrast, the amount of L1 protein in lesioned nerves of lines 3427 and 3426 increased by approximately 30% when compared with the unlesioned contralateral side. The expression level of L1 in line 3426 was approximately 35% and 25% higher than in the line 3427 for unlesioned and lesioned optic nerves, respectively.

20

#### **EXAMPLE 11**

##### ***In vivo* regrowth of axons in the optic nerve**

6-8 week old GFAP-L1 transgenic mice and wild type mice were crushed intraorbitally and, after 14 days, traced with a fluorescein-labeled biotin ester to mark retinal ganglion cell axons by anterograde labeling. Results are shown in Figures 11 and 12. Each point represents one animal.

#### **EXAMPLE 12**

**Identification of the border between fibronectin type III homologous repeats 2 and 3 of the neural cell adhesion molecule L1 as a neurite outgr wth promoting and signal transducing domain**

To determine the domains of neural cell adhesion molecule L1 involved in neurite outgrowth, monoclonal antibodies against L1 were generated and their effects on neurite outgrowth of small cerebellar neurons in culture investigated. When the eleven antibodies were coated as substrate, only antibody 557.B6, which recognizes  
5 an epitope represented by a synthetic peptide comprising amino acids 818 to 832 at the border between the fibronectin type III homologous repeats 2 and 3, was as potent as L1 in promoting neurite outgrowth, increasing intracellular levels of  $Ca^{2+}$  and stimulating the turnover of inositol phosphates. These findings suggest that neurite outgrowth and changes in these second messengers are correlated. Such a  
10 correlation was confirmed by the ability of  $Ca^{2+}$ -channel antagonists and pertussis toxin to inhibit neurite outgrowth on L1 and antibody 557.B6. These observations indicate for the first time a distinct site on cell surface-bound L1 as a prominent signal transducing domain through which the recognition events appear to be funnelled to trigger neurite outgrowth, increase turnover of inositol phosphates and  
15 elevate intracellular levels of  $Ca^{2+}$ .

### **EXAMPLE 13**

#### **L2/HNK-1 immunoreactivity in reinnervated peripheral nerve: preferential expression of previously motor axon-associated Schwann cells**

The carbohydrate epitope L2/HNK-1 (hereafter designated L2) is expressed in the  
20 adult mouse by myelinating Schwann cells of ventral roots and muscle nerves, but rarely by those of dorsal roots or cutaneous nerves. Since substrate-coated L2 glycolipids promote outgrowth of cultured motor but not sensory neurons, L2 may thus influence the preferential reinnervation of muscle nerves by regenerating motor axons *in vivo*.

25 Therefore, the influence of regenerating axons on L2 expression by reinnervated Schwann cells was analyzed by directing motor or sensory axons into the muscle and cutaneous branches of femoral nerves of eight-week-old mice. Regenerating axons from cutaneous branches did not lead to immunocytochemically detectable L2 expression in muscle or cutaneous nerve branches. Axons regenerating from



muscle branches led to a weak L2 expression by few Schwann cells of the cutaneous branch, but provoked a strong L2 expression by many Schwann cells of the muscle branch. Myelinating Schwann cells previously associated with motor axons thus differed from previously sensory axon-associated myelinating Schwann cells in their ability to express L2 when contacted by motor axons. This upregulation of L2 expression during critical stages of reinnervation may provide motor axons regenerating into the appropriate, muscle pathways with an advantage over those regenerating into the inappropriate, sensory pathways.

#### **EXAMPLE 14**

##### **10 L1 in consolidation of memory for a passive avoidance task in the chick**

Training day-old chicks on a one trial passive avoidance task, in which they learn to suppress their tendency to peck at a small bright bead if it is coated in the bitter-tasting methylanthranilate, results in a time-dependant cellular and molecular cascade culminating in the remodelling of pre- and post-synaptic elements in two discrete regions of the forebrain, the intermediate medial hyperstriatum ventrale (IMHV) and Lobus parolfactorius (LOP) (Rose (1991) *Trends In Neurosciences* 14:390-397). The cascade involves two distinct waves of glycoprotein synthesis, as evidenced by enhanced fucose incorporation, occurring in both IMHV and LPO at varying times following training. Both waves are necessary for long-term (that is, 24 hours plus) memory retention for the avoidance tasks, in which amnesia is evidenced by chicks, which would otherwise avoid the previously bitter bead, pecking at a dry bead on test.

Given the role of L1 in mediating cell-cell contact, the present study was undertaken in order to determine if L1 is amongst the learning-associated glycoproteins participating in either or both waves of glycoprotein synthesis, and is necessary for memory formation. If so, antibodies to L1 administered at an appropriate time relative to training should prevent the synaptic remodelling necessary for long term memory and therefore produce amnesia for the task. Similarly, if the extracellular domains of the L1 molecule play a part in the

recognition and adhesion processes which are required for synaptic remodelling and stabilization, exogenously applied extracellular domain fragments which will bind homophilically to the endogenous molecule might disrupt this process.

#### **Antibodies and Fragments**

- 5 Polyclonal antibodies were prepared in rabbits by immunization with immuno-affinity purified L1 (Ng-CAM, 8D9) following an established immunization procedure (Rathjen et al. (1984)). L1 was isolated from one-day old chicken brains using an 8D9 monoclonal antibody (Lagenaur and Lemmon (1987) *Proc. Nat'l Acad. Sci. USA* 84:77533-7757) column again using established procedures
- 10 (Rathjen et al. (1984)). Antibodies were isolated from the serum obtained after the third immunization using Protein G Sepharose (Pharmacia LKB) according to the manufacturer's instructions. Recombinantly expressed fusion proteins in *E. coli* representing the six immunoglobulin-like (Ig-I-VI) and five fibronectin type III homologous repeats (FN1-5) were prepared as described by Appel et al (1993).
- 15 **Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots of chick subcellular fractions**
- Fifty µg of protein from brain homogenate, from crude membranes, from a soluble fraction (Burchuladze et al. (1990) *Brain Res.* 535:131-138) and from postsynaptic densities (Murakami et al. (1986) *J. Neurochem.* 46:340-348), all from day-old
- 20 chicken brains were separated by SDS-PAGE under reducing conditions on a 5-15% polyacrylamide gradient gel (Laemmli (1970) *Nature* 227:146-148), whereafter they were transferred to nitrocellulose according to the method of (Burnette (1981) *Anal. Biochem.* 112:195-203). After overnight incubation with L1 antibodies at a dilution of 1:1,000 in Tris-buffered saline, pH 7.2, containing 5%
- 25 defatted milk powder, immunoreactive bands were detected according to previously described methods (Scholey et al. (1993) *Neurosciences* 55:499-509).

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#### **Training and testing procedures**

Day-old Ross chunky chicks of both sexes, hatched in incubators were place in pairs in small pens, pretrained to peck at small (2.5 mm) white beads and then trained on a larger (4mm) chrome bead coated with methylantranilate as described by Lossner and Rose ((1983) *J. Neurosciences* 41:1357:1363). Birds which pecked the bitter bead evinced a stereotyped disgust response, shaking their heads vigorously and backing away from the bead. Twenty four hours following training, each animal was tested by the presentation of a dry chrome bead identical to the one used in training. Retention of passive avoidance learning was indicated in animals avoiding the test bead. In each replication of this protocol, 24-36 chicks were trained and tested. More than 80% of trained, uninjected chicks normally avoid the bead on test under these conditions, though there is sometimes a slight reduction in avoidance in saline injected birds. By contrast, birds which are trained on a water-coated bead peck the dry bead avidly on test, and their avoidance score is rarely above 5-10%. All training and testing was routinely carried out by an experimenter blind as to the prior treatment of the animals.

### Injections

L1 antibodies, FN1-5 and Ig I-VI fragments were dialyzed overnight against 0.9% saline and the concentration adjusted to 1 mg/ml for L1 and 250 µg/ml for the fragments. Chicks received bilateral intracranial injections into the intermediate medial hyperstriatum ventrale (IMHV) of 10 µl L1 antibodies per hemisphere; control animals received similar injections of saline. Accurate delivery into the IMHV was received by the use of a specially designed head holder and sleeved Hamilton syringe (Davis et al. (1982) *Pharm. Biochem. Behav.* 17:893-896). Chicks receiving this injection volume of either saline or antibodies prior to training or testing showed no overt behavioral effects, pecking the bead accurately during training. The large extracellular volume of the brain of the newly hatched chick means that injections of this size are well-tolerated, and can be achieved without leakage. A previous report has demonstrated (Scholey et al. (1993)) that there is a slow diffusion of antibody from the injection site in the hours following injection. The accuracy of placement of the injection was routinely monitored by

visual inspection of the brains post-mortem. In each replication of the experiment, a balanced group of saline and antibody or fragment-injected chicks were employed. In the L1 experiment, groups of chicks were injected with saline or antibody at one of eight time points relative to training; 2 hours or 30 minutes pre-training, or +1 hour, +3 hours, +4 hours, +5.5 hours, +8 hours or +12 hours post-training. On the basis of previous observations, it was predicted that any effects would be observed in birds injected at either 30 minutes prior or 5.5 hours post-training, and the numbers of replications at these time points were accordingly greater (N=17, 28, 17, 19, 18, 21, 19 and 18 respectively for antibody injections). L1 fragments FN1-5 and Ig I-IV were injected at either -30 minutes or +5.5 hours and retention tested at 24 hours. Retention in groups of saline and L1-antibody or L1-fragment-injected chicks was compared statistically by  $\chi^2$ . Results are shown in Figures 13 and 14.

#### EXAMPLE 15

##### 15 **Involvement of L1 and NCAM in long term potentiation**

Transverse hippocampal slices (400  $\mu$ m) from halothane-anaesthetized male Wistar rats (180-220g) were prepared using standard techniques. Slices were maintained in an interface chamber and initially allowed to recover for 45 min. in a hyperosmolar (320 mOsm/kg) artificial cerebrospinal fluid (ACSF) at room temperature. The bath temperature was then raised to 30°C and the medium was changed to a normotonic ACSF (307 mOsm/kg) containing (in mM): NaCl, 124.0; KCl, 2.5; MgSO<sub>4</sub>, 2.0; CaCl<sub>2</sub>, 2.5; KH<sub>2</sub>PO<sub>4</sub>, 1.25; NaHCO<sub>3</sub>, 26.0; glucose, 10; sucrose, 4; bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> (pH 7.4); perfusion rate: 0.75 ml/min. The Schaffer collateral/commissural fibers were stimulated by twisted platinum-iridium wires (50  $\mu$ m diameter) placed in the stratum radiatum of the CA1 region. Test stimuli consisted in monophasic impulses of 100  $\mu$ s duration every 30 seconds and the stimulus strength was adjusted to obtain 30% of the maximal EPSP amplitude (maximal EPSP without superimposed population spike). EPSP's were recorded from the CA1 stratum radiatum by means of 2 glass micropipettes (2 M

NaCl, 1-5 M $\Omega$ ) positioned about 300  $\mu$ M apart from the stimulation electrode on each side.

After stable recording for at least 15 minutes, antibodies or protein fragments were ejected onto the CA1 dendritic field in the vicinity (50-75  $\mu$ m) of one recording  
5 electrode (the one carefully adjusted at 30%) by using a modified microinjection system (Nanoliter injector, WPI) continuously delivering 5 nl every 10 seconds up to the end of the experiment unless otherwise indicated. A wash-out of the antibodies with subsequent induction of LTP was not possible for evident reasons, but it was verified whether LTP could be induced within each slice by recording  
10 from the second electrode where no antibodies were applied. Although the tip of the ejection micropipette did not penetrate the slice, a small reduction in the EPSP amplitude was sometimes observed when the ejection was started. This volume artifact was independent of the nature of the ejected material. Proteins were dialyzed against 20 mM PBS at pH 7.4 unless otherwise indicated and  
15 concentrations referred to the pipette concentration.

Twenty minutes after initiating the microejection, LTP was induced with a theta burst stimulation (TBS) paradigm consisting of three trains spaced by 4 seconds; each train consisted of ten high frequency bursts of 5 pulses at 100 Hz and the bursts were separated by 200 ms (Reichardt et al. (1991) *Annu. Rev Neurosci.*  
20 14:531-570). Duration of the stimulation pulses was doubled during TBS. Induction of LTP could be totally prevented by perfusion of 10  $\mu$ M D(-)-2-amino-5-phosphonopentanoic acid (D-AP5; Tocris). Whole cell recordings were obtained from CA1 neurons using the "blind" patch clamp method with an EPC-9 patch clamp amplifier. The bath temperature was 30°C. Patch electrodes were pulled  
25 from 1.5 mm OD borosilicate glass and had resistances between 3 and 8 M $\Omega$ . The pipettes were neither fire polished nor coated. The electrodes were routinely filled with a solution containing (in mM): potassium gluconate, 129; KCl, 5; MgCl<sub>2</sub>, 1; CaCl<sub>2</sub>, 1; N-(1-hydroxyethyl)-piperazine-N'-(2-ethanesulphonic acid) (HEPES), 5; 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), 5; Na-ATP,

- 10 and Na-GTP, 0.3. with pH adjusted to 7.3 using KOH. Series resistance was not compensated. Responses were sampled as an average of three to four signals, either printed out for visual analysis, or stored on disk for further analysis. Statistical evaluations were performed by analysis of variances with planned
- 5 comparisons and contrast analysis; time was considered as a dependent variable with one level of repeated measures. Anti-L1 (Rathjen et al. (1984)), anti-Ig I-VI (Hynes et al. (1992)) and anti-liver membranes antibodies (Linder et al. (1983)) were produced as previously described. Results are shown in Figure 15.
- 10 Ig-like domains I-VI and FN type III homologous repeats I-V of L1 were expressed in bacteria and purified as described (Hynes et al. (1992)). Antibodies to NCAM and axonin-1 were produced as described (Larson et al. (1986) *Science* 232:985-988; Bailey et al. (1992) *Science* 256:645-649). Production of oligomannosidic glycopeptides from ribonuclease B and control glycopeptides from asialofetuin have been described (Larson et al. (1986)). Results are shown in Figure 16.
- 15 NMDA receptor-mediated EPSP's were isolated by applying 30  $\mu$ M of the non-NMDA blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris) starting 20 minutes prior to the application of antibodies or glycopeptides. At the end of each experiment, it was verified that D(-)-2-amino-5-phosphonopentanoic acid (D-AP5; 30  $\mu$ M; Tocris) completely suppressed these responses. Results are shown in
- 20 Figure 17.

### EXAMPLE 16

#### **L1 exerts neuropreservative effect**

- An experiment was performed to further elucidate the activity of L1 with CNS nerve tissue. Specifically, aliquot samples of mouse mesencephalon cells were
- 25 plated and cultured on four separate plates having media prepared as follows: the first control plate was coated with poly-L-lysine alone; a second plate was coated with poly-L-lysine and L1; a third control plate was coated with poly-L-lysine and laminin; and a fourth plate was coated with poly-L-lysine, laminin and L1. All

plates received aliquot amounts of cells and were incubated under identical conditions. After 7 days, the plates were all stained for the presence of dopamine and thereafter observed. The plates that were coated with L1 exhibited a growth of 200% to 400% greater than the controls. The plates coated with laminin exhibited 5 greater neurite outgrowth, but not more cells than those coated with L1. The results demonstrate and suggest that L1 exerts a profound neuropreservative effect, as cell viability measured by numbers of cells grown was dramatically increased over controls.

#### **EXAMPLE 17**

10 **Soluble L1 (L1-Fc) is functionally active and is a potent agent in neuronal survival**

Soluble L1 was made in COS cells as a recombinant L1-Fc fusion protein by the procedure described in *Neuron* 14:57-66, 1995. The recombinant protein was purified by Protein A affinity chromatography, and was used either as a substrate 15 coated onto plastic or as a soluble molecule added to the culture medium at approximately 1-10 µg/ml. Neurite outgrowth and survival of mesencephalic neurons from day 17 rat embryos were examined in culture after 7 days *in vitro* maintenance. Dopaminergic neurons were recognized by immunostaining for dopamine-β-hydroxylase (DBH) and quantified using IBAS morphometric 20 equipment. Cultures with added soluble L1-Fc were maintained on poly-DL-ornithine (PORN) and substrate-coated L1-Fc was added on top of previously coated PORN (under conditions described in Appel et al, *J. Neuroscience* 13:4764-4775, 1993). NCAM-Fc was used as a control.

Table - Survival and neurite outgrowth of DBH<sup>+</sup> neurons after 7 days *in vitro*

|                     | number of neurons <sup>-</sup> | length of neurites <sup>--</sup> |
|---------------------|--------------------------------|----------------------------------|
| substrate-coated L1 | 129 ± 20                       | 179 ± 40                         |
| soluble L1          | 98 ± 7                         | 135 ± 27                         |
| PORN only (control) | 14 ± 2                         | 37 ± 9                           |

5 Mean values are from at least three independent experiments ± SEM

- The numbers are from a unit field

-- The lengths of all neurites (total neurite length) per neuron was determined (in μm)

Recognition among neural cells is an important prerequisite for the development of  
 10 a functioning nervous system. Recognition molecules are expressed at the cell  
 surface, where they mediate interaction between neighboring cells, like cadherins,  
 or between the cell surface and the extracellular matrix, like integrins (Takeichi,  
 1991; Ruoslahti, 1988; Hynes, 1992). The most prominent family of recognition  
 molecules comprises immunoglobulin (Ig)-like domains. The Ig-like domains  
 15 reflect a common ancestry of immunoglobins and cell adhesion molecules, both of  
 which are involved in specific recognition events (Edelman, 1970). In the nervous  
 system the Ig superfamily comprises by now more than two dozen distinct  
 molecules. Some Ig-like domain containing molecules have multiple functions  
 within the extracellular domain: Receptors for cytokines and neurotrophins have  
 20 high affinity receptive functions as well as recognition properties (Tannahill et al.,  
 1995; Pulido et al., 1992). The three-dimensional structure of Ig-like domains is  
 similar to FN-like repeats (Main et al., 1992; Leahy et al., 1992), which are also  
 structural motifs in several extracellular matrix molecules, such as fibronectin,  
 members of the tenascin family, and others (Williams and Barclay, 1988; Baron et  
 25 al., 1992; Erickson, 1993). Neural recognition-molecules-of-the-Ig-superfamily have  
 characteristic temporal, spatial, and cell-type specific expression patterns (for  
 reviews, see Edelman, 1988; Schachner, 1991, 1994; Rathjen and Josseli, 1991;



Rutishauser, 1993). Recognition molecules of this family are functionally overlapping in that all promote cell adhesion and neurite outgrowth. Some recognition molecules are strongly homophilic, i.e. self binding partners, whereas others are predominantly heterophilic, i.e. they bind to non-self partners which often comprise other members of the Ig superfamily or extracellular matrix molecules (Brümmendorf and Rathjen, 1993, 1994). Present knowledge of the functional properties of the individual Ig-like domains and/or FN-like repeats of neural recognition molecules indicate both distinct, and overlapping functional properties in recognition, neurite outgrowth, and repulsion (Gennarini et al., 1991; Frel et al., 1992; Taylor et al., 1993; Appel et al., 1993, 1995; Pesheva et al., 1993; Feisenfeld et al., 1994; Hoim et al., 1995).

Among neural recognition molecules of the Ig superfamily, the family of molecules related to the neural recognition molecule L1 shows striking similarity in function and structure. They are potent neurite outgrowth promoters and are expressed relatively late during development, mostly at the state when axogenesis occurs. They are predominantly expressed by neurons, although some members of the L1 family are also present on neurite outgrowth promoting glial cells (Martini and Schachner, 1986; Bixby et al., 1988; Seilheimer and Schachner, 1988).

In the experiments that follow, another member of the L1 family is identified and characterized, that is designated a close homolog of L1 (CHL1). It contains six Ig-like domains and FN-like repeats, of which four are highly homologous to the FN-like repeats of other L1 family members. The partial FN-like repeat localizes to the membrane-adjacent region of the molecule, which is the most variable region among L1 related molecules. Other features of CHL1 shared with members of the L1 family are its predominant and developmentally late expression in the nervous system, and its high level of N-glycosylation, including expression of the HNK-1 carbohydrate.

**EXAMPLE 18****MATERIAL AND METHODS****Animals**

ICR mice and Wistar rats were used for tissue preparations.

**5 Antibodies**

Polyclonal antibodies directed against the recombinantly expressed extracellular part of CHL1 (amino acids 499-1063 (Figures 18 and 19)) and L1 (amino acids 126-1981 (Appel et al., 1993)) were raised in rabbits as described (Rathjen and Schachner, 1984). To raise antibodies against CHL1 200 µg of purified peplite  
10 was injected into rabbits followed by four additional injections of 100 µg in intervals of three weeks. L1 antibodies were concentrated from serum by ammonium sulfate precipitation (13.5 mg/ml). Monoclonal rat antibody 412 was used to identify the HNK-1 carbohydrate epitope (Kruse et al., 1984). Monoclonal antibodies against glial fibrillary acidic protein (GFAP) were obtained from  
15 Boehringer (Mannheim). The monoclonal antibodies to the O1 antigen(s) has been described (Sommer and Schachner, 1981).

**Purification of neural adhesion molecules**

L1, N-CAM, and MAG were immunoaffinity purified from detergent extracts of crude membrane fractions from adult mouse brain using monoclonal antibody  
20 columns (Rathjen and Schachner, 1984; Falssner et al., 1985; Poltorak et al., 1987).

**cDNA libraries and screening**

Preparation of the λgt11 library derived from poly(A)<sup>+</sup> RNA of brains from 8-day-old mice and screening of this library with immunoaffinity purified polyclonal L1 antibodies were performed as described (Tacke et al., 1987). To obtain longer  
25 cDNA clones, a new DNA library was constructed: RNA was purified from brains of 6 to 14-day-old mice by the guanidinium-thiocyanate/acid phenol method (Chomezynski and Sacchi, 1987). Poly(A)<sup>+</sup> RNA was enriched by two subsequent

passages over an oligo(dT)-cellulose column (Sambrook et al., 1989). Eight micrograms of poly(A)<sup>+</sup> RNA were used for synthesis of oligo(dT)-primed double-stranded cDNA using a cDNA synthesis kit (Amersham). The cDNA was size-selected and ligated into the plasmid pXMD1 with DraIII-adaptors containing a Sall site (Kluxen et al., 1992). For propagation and amplification of the library, *E. coli* strain TOP10 (Invitrogen, Netherlands) was used. For screening, aliquots were directly plated onto Nylon membranes (BIODYNE™ Pall) with a density of about  $2 \times 10^4$  bacteria/filter ( $138 \text{ cm}^2$ ). Replica filters were incubated overnight at 37°C. Subsequently, the bacteria were lysed (0.5M NaOH; 1.5M NaCl), filters were neutralized (3M NaCl; 0.5M Tris-HCl pH 8.0), washed in a 2xSSC, air-dried, and baked for 2 hr. at 80°C. The Nylon membranes were prehybridized, hybridized with a 1 kb fragment (HincII/KpnI) of CHL1 (derived from the  $\lambda$ gt11 library) redioiabelled by random-priming (Boehringer Mannheim) according to the manufacturer's protocol, washed under high-stringency conditions at 42°C, and then exposed to X-ray film as described elsewhere (Sambrook et al., 1989). Six positive clones were further characterized by restriction mapping and sequencing according to standard protocols (Sambrook et al., 1989). One clone (pX#2) containing a 4.43 kb CHL1 insert was used for further analysis.

#### DNA Sequencing and sequence analysis

Nucleotide sequences were determined by the dideoxy-chain-termination method (Sanger et al., 1977) using double-stranded DNA as a template for T7 DNA polymerase (Pharmacia) and synthetic oligonucleotides as primers. cDNA sequences were assembled and analyzed with the DNASTAR program (DNASTAR, Inc., London). Unless otherwise indicated, amino acid sequences were aligned by the Jolun Hein method (Hein, 1990) (gap penalty = 11, gap length penalty = 5, K tuple = 2).

#### Comparison of protein sequences

To calculate a similarity index (%) for comparison of the distances between conserved amino acid residues, several distinct proteins containing six Ig-like

domains and at least four FN-like repeats were aligned at conserved amino acid residues in the Ig-like domains (cysteines which refer to S-S bridges) and in the FN-like repeats (tryptophan and tyrosine/phenylalanine). The number of amino acid residues between these conserved positions was determined. This is referred to as the consensus distance. The mean value of the distances, i.e. the consensus distance and standard deviation (SD) among L1 family members were calculated. SD values were rounded up to the next integer. The distance for each protein was compared to the mean distance and considered as match if the distance value equaled the mean value  $\pm$  the SD (= consensus distance). The number of matches to the 19 consensus distances was calculated for each individual protein (similarity index = number of matches / 19 x 100). For example: In the CHL1 protein 16 distance values match to the consensus distances, while three of all criteria did not match. This leads to a similarity index of 16 / 19 or 84% for CHL1.

#### **EXAMPLE 19**

##### **15 Cell culture and expression of CHL1 and L1 in COS-1 cells**

The 4.43 kb insert of clone pX#2 was ligated into SalI digested pXMD1 (Kluxen et al., 1992, Kluxen and Lobbert, 1993). A subfragment (EcoRI (plasmid polylinker)/PvuII bp 4048) of the mouse L1 cDNA (Moos et al., 1988) was treated with T4 DNA polymerase and ligated into pXMD1.

20 COS-1 cells were maintained in DMEM (0.1% glucose) supplemented with 10% (v/v) fetal calf serum at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. DEAE dextran-mediated DNA transfection was performed as described (Kluxen et al., 1992) with some modifications. Briefly, the cells were seeded at about 10,000 cells/cm<sup>2</sup>. One day later, after two washes with DMEM (0.45% glucose), the medium was replaced by transfection solution composed of DMEM supplemented with 10% (v/v) Nu-serum (Becton Dickinson, Switzerland), 0.4 mg/ml (w/v) DEAE-dextran (Pharmacia), 50 µM chloroquine, and 1.25 µg/ml DNA (4 ml per 10 cm dish). The cells were incubated 4-hr at 37°C and 5% CO<sub>2</sub>. Then the medium was removed and the cells were incubated for 2 min in phosphate-buffered

- saline (pH 7.3) containing 10% dimethylsulfoxide (v/v). After two washes with DMEM<sub>1</sub> (0.45% glucose), DMEM supplemented with 10% (v/v) fetal calf serum and 20 µg/ml gentamycin was added and the cells were incubated in this medium. 24 hr later cells were detached by incubation with 0.01% trypsin and 0.0004% EDTA in Hanks' balanced salt solution (HBSS) for 5 min at 37°C, replated for immunocytochemistry at a density of about 20,000 cells/cm<sup>2</sup> in 24-well plates (Falcon) containing poly-L-lysine coated glass coverslips (11 mm in diameter), and incubated for an additional 24 hr. For Western blot analysis the cells were replated on tissue culture dishes and incubated for an additional 48 hr.
- 10 PC12 cells were maintained in DMEM with 10% (v/v) fetal calf serum and 5% (v/v) horse serum on collagen coated tissue culture dishes. For induction of the cells with nerve growth factor (NGF) the medium was removed from monolayers at about 50% confluency and replaced with medium of reduced serum content (5% horse serum) supplemented with 100 ng/ml 7s-NGF (Sigma, Switzerland). After two days of incubation the cells were detached by incubation with 0.1% trypsin and 0.04% EDTA, collected and subjected to RNA extraction.

Primary cultures of astrocytes were prepared according to McCarthy and De Vellis (1980) with modifications (Guénard et al. 1994) and used for immunostaining after one to two weeks *in vitro*. Primary cultures of oligodendrocytes were prepared as described by Laeng et al. (1994) and maintained *in vitro* for 12 days.

### **EXAMPLE 20**

#### **Production of antisense RNA**

- The 4.43 kb insert of clone pX#2 was ligated into Sall digested pBS II SK. (Stratagene) followed by deletion of an ApaI (vector)/AvrII (bp 3330 (Figure 18)) fragment to obtain the cDNA fragment of CHL1 encoding the extracellular part of the protein (see Figure 18 and 19). A similar construct for L1 was prepared by ligation of an EcoRI (plasmid polylinker)/EcoNI (bp 3304) fragment of the L1 cDNA (Moos et al., 1988) treated with T4 DNA polymerase and ligated into SmaI

digested pBS II SK-. The plasmids were digested with XbaI and used for synthesis of  $^{32}\text{P}$ -labeled antisense RNA with T7 RNA polymerase as described (Melton et al., 1984).

#### Northern blot analysis

- 5 Poly (A)<sup>-</sup> mRNA was prepared from different tissues of neonatal and 9-day-old mice using the Oligotex<sup>TM</sup> Direct mRNA-Method (QIAGEN Inc., Düsseldorf, Germany) following the manufacturer's instructions. Poly (A)<sup>-</sup> mRNA and RNA marker (RNA ladder, GIBCO/BRL) were subjected to electrophoresis on a 0.8 % formaldehyde/agarose gel and subsequently transferred to Hybond-N membrane
- 10 (Amersham) by capillary transfer (Southern, 1975) in 20x SSC. After UV crosslinking (UV-Stratalinker® 1800, Stratagene, La Jolla, CA), the amount of RNA transferred and bound to the membrane was controlled by methylene blue staining (Sambrook et al., 1989). Following prehybridization for 2 hr at 65°C, the membrane was hybridized overnight using CHL1- and L1- specific  $^{32}\text{P}$ -labeled
- 15 antisense RNA probes in hybridization buffer (5xSSC, 2.5xDenhart's solution, 50 mM Na<sub>2</sub>PO<sub>4</sub> (pH 6.5), 0.1% SDS, 1mM EDTA, 2 µg/ml salmon sperm DNA, 50% formamide) at 65°C. The filter was then washed three times at 65°C in 0.1xSSC, 0.1% SDS for 1 hr and exposed to X-ray film.

#### EXAMPLE 21

##### 20 **Expression and purification of recombinant CHL1 protein in *E. coli***

- A 1.7-kb cDNA-fragment of CHL1 (MscI; bp 1791 (which originates from the vector cloning site and the 5' end of the λgt11 derived CHL1 clone) and BsmAI; bp 3494) encoding the 6th Ig-like domain (IgV1) and FN-like repeats 1.4.5 (see Figures 18 and 19b) was subcloned into the unique BamHI restriction site of the
- 25 pET-vector (Studier and Moffatt, 1986). The correct sequence of the plasmid was confirmed by sequencing. *E. coli* strain BL21 (DE3) was transformed with this plasmid. Expression and purification by anion exchange chromatography of the recombinant protein were performed according to Appel et al. (1993). SDS-PAGE

and Coomassie staining showed a major band at the expected molecular weight (70 kD) which contained at least 80% of the total protein (not shown).

#### Tissue fractions

Detergent lysates of whole tissue were prepared by homogenization of tissues in 40 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5mM EGTA, 1mM phenylmethanesulfonylfluoride (PMSF), 1% Triton X-100 and maintained at 4°C for 3 hr under constant stirring. The soluble fraction was separated from insoluble material by centrifugation at 100,000 g.

For preparation of detergent lysates of membrane fractions, tissues were homogenized in 1 mM NaHCO<sub>3</sub> (pH 7.9), 0.2 mM CaCl<sub>2</sub>, 0.2 mM MgCl<sub>2</sub>, 1 mM spermidine, 5 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 1 mM PMSF, and 0.5 iodoacetamide at 4°C. Membrane and soluble fractions were then separated and the membrane pellet was resuspended in solubilization buffer (20 mM Tris-HCl (pH 7.9), 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 5 µg/ml Aprotinin, 10 µg/ml soybean trypsin inhibitor, 1 mM PMSF, and 0.5 mM iodoacetamide).

Transiently transfected COS-1 cells were washed twice with HBSS and incubated with 1 mM EDTA in HBSS for 10 min at 37°C. The cells were then detached with a fire polished Pasteur pipette and collected by centrifugation at 200g for 10 min at 4°C. The cells were lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM iodoacetamide, 1 mM PMSF, and 1% MP-40 and the supernatant was cleared by centrifugation (13000g). Protein determinations were performed as described by Bradford (1976).

#### Western blot analysis

Proteins were separated by SDS-PAGE (Laemmli, 1970) on 8% or 10% slab gels under reducing conditions and transferred to nitrocellulose filters (0.45 µm, BA 85; Schleicher & Schuell, Dassel, Germany) for immunodetection according to Faissner

et al. (1985), using CHL1 antiserum (diluted 1:500, 1:10000 for ECL), L1 polyclonal antibodies (diluted 1:1000, 1:15000 for ECL), or monoclonal antibody 412 (diluted 1:1000, 1:10000 for ECL) and alkaline phosphatase-coupled secondary anti-rabbit or anti-rat IgG. Bound antibodies were either detected by the enhanced  
5 chemiluminescence (ECL) method according to the manufacturer's instructions using ECL Western blotting detection reagents (Amersham) and X-ray films, or by using BCIP and NBT as chromogenic substrates.

### **EXAMPLE 22**

#### **Enzyme-linked immunosorbent assay**

- 10 The enzyme-linked immunosorbent assay (ELISA) was performed as described by Husmann et al. (1992) with the exception that proteins were coated at concentrations of 100 ng/ml. CHL1 antiserum was used in several dilutions between 1:250 to 1:2X10<sub>6</sub>.

#### **Deglycosylation of CHL1**

- 15 Detergent lysates of brain tissue homogenate (200 µl, 6 mg/ml protein concentration) from seven-day-old mice were separated into a soluble fraction and insoluble material by centrifugation (see Tissue fractions) and incubated with 0.5 units N-glycosidase F or 2.5 units O-glycosidase, or both enzymes at these concentrations according to the manufacturer's instructions (Boehringer Mannheim,  
20 Germany). The lysates were resolved by SDS-PAGE on 10% gels. The proteins were transferred to nitrocellulose and incubated with CHL1 antiserum (1:500 diluted) directed against the recombinant CHL1 protein fragment (see Figure 18).

#### **Immunoprecipitation**

- The soluble fraction of detergent lysates of brain tissue homogenate (300 µl, 5  
25 mg/ml protein concentration) from nine-day-old mice (see Tissue fractions) was incubated with 10 µl. CHL1 antiserum or polyclonal antibodies against L1 overnight at 5°C in 5-ml buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA) containing 1% NP40 and 30 µl of G-Sepharose (Pharmacia/LKB). After



sequentially washing the buffers containing 0.1% NP40, 0.05% SDS, and then 20 mM Tris-HCl (pH 7.4), the Sepharose beads were boiled for 10 min in 5x sample buffer (250 mM Tris-HCl (pH 6.6), 10% SDS, 50% glycerol, 0.5% bromophenol blue, 25% B-mercaptoethanol) and the supernatant was resolved by SDS-PAGE on 5 10% gels. The proteins were transferred to nitrocellulose and detected with polyclonal antibodies against L1, CHL1 antiserum, or monoclonal antibody 412 by Western blot analysis.

#### **Indirect immunofluorescence**

For cell surface staining (Schnitzer and Schachner, 1981) CHL1-, and mock (vector only)- transfected COS-1 cells plated on coverslips were incubated for 30 min at 10 room temperature with primary antibody (CHL1 antiserum (1:100 diluted) or L1 polyclonal antibodies (1:200 diluted)) in DMEM containing 10% fetal calf serum, 10 mM Hepes (pH 7.3), and 0.02% NaN<sub>3</sub>, and then with secondary antibody. After 15 innunostaining, the cells were fixed with 4% paraformaldehyde in phosphate buffered saline (pH 7.3) and mounted in Moviol (Hoechst) containing 2.5% potassium iodide. For double-immunofluorescence staining of astrocytes and oligodendrocytes, incubation of primary antibodies to cell surface antigens was performed as described for transfected COS-1 cells. Subsequently, incubation of cells with primary antibodies against intercellular antigens was performed after 20 permeabilizing the cells with methanol at -20°C.

### **EXAMPLE 23**

#### ***In Situ Hybridization***

To generate digoxigenin-labelled antisense cRNA probes of equal size from corresponding parts of CHL1 and L1 the same constructs as for Northern blot 25 analysis were used. Sense probes were generated from similar constructs with the inserts in opposite direction. All the cRNA probes were generated using T7 RNA polymerase followed by an alkaline treatment to obtain an average fragment length of 250 nucleotides. *In situ* hybridization was performed as described (Bartsch et al., 1992; Dorries et al., 1994).

## RESULTS AND DISCUSSION

### Identification of CHL1 cDNA

Screening of a  $\lambda$ gt11 expression library for cDNA clones encoding the cell adhesion molecule L1 with polyclonal antibodies raised against brain-derived immunopurified L1 (Tacke et al. 1987) identified the clone 311. It contained a partial cDNA homologous to L1 (34.1% according to Lipman and Pearson (1985)) and an open reading frame of 2112 base pairs (bp) coding for 704 amino acids including the cytoplasmic part. To isolate full length cDNA clones, a DNA fragment of this clone was used for screening a different cDNA library. Six independent clones were isolated. Two clones contained 4.2 and 4.4 kb inserts comprising the entire coding region of a close homolog of L1 (CHL1). The clone containing the 4.4 kb insert was further investigated.

### DNA and deduced amino acid sequences and structural features

The 4.4 kb insert encodes a 5' untranslated region of 295 bp, an open reading frame of 3627 bp, and a 3' untranslated region of 518 bp (Figure 18). Although there is an oligo(A) tract at its 3' terminus, a clear consensus polyadenylation signal upstream of this sequence is missing. The flanking sequences of the AUG start codon (position 296, Figure 18) do not conform to the optimal consensus sequence for initiation of translation (Kozak, 1987). However, this AUG is taken as the start codon for translation based on two lines of evidence. It is preceded upstream by stop codons in all three reading frames and is followed by a potential signal sequence with a cleavage site predicted after residues 24 or 25 (scores of 8.65 and 6.40, respectively, according to the algorithm of von Heijne (1986)) (Figure 18).

Translation of the open reading frame yields a protein of 1209 amino acids with a calculated molecular mass of 134.9 kD and features characteristic of an integral membrane glycoprotein. The putative extracellular domain is composed of 1081 amino acids, with 18 potential sites for N-glycosylation (Figures 18 and 19a) and

more than 60 potential O-glycosylation consensus sites (not shown) (Pisano et al., 1993), followed by a transmembrane domain of 23 amino acids, as judged by hydropathy analysis according to Kyte and Doolittle (1982) (Figure 19c). This domain is flanked at its N-terminal end by a polar residue and at its C-terminal end  
5 by a basic amino acid, consistent with a stop transfer signal (Figure 18). The intracellular region is composed of 105 amino acid residues.

The extracellular region contains the two major structural motifs of repeated domains that are characteristic of the L1 family: a 685 amino acid stretch with  
10 homology to Ig-like domains and a 472 amino acid stretch with homology to FN-like repeats (Figures 1 and 2a). All of the six Ig-like domains contain the characteristic pair of cysteine residues located at 47-54 amino acids apart from each other (Figure 19). A conserved proline (except in the sixth Ig-like domain) at the end of  $\beta$ -strand B in conjunction with a C2-type cluster of conserved amino acids around the second cysteine residue in each domain (DXGXYXCXAXN) assign the  
15 Ig-like domains to the C2-set (Williams and Barclay, 1988). Between the Ig-like domains and the membrane spanning region are four domains that are homologous to the FN-like repeats in fibronectin (Kornblihtt et al., 1985). Each of these domains of approximately 100 amino acids contains the highly conserved tryptophan (except for the first FN-like repeat) and tyrosine/phenylalanine residues  
20 in the N- and C-terminal regions, respectively. Interestingly, the fifth FN-like repeat is, in contrast to the other members of the L1 family, only a rudimentary one-half FN-like repeat (Figure 18). Whether this half FN-like repeat represents one of several alternatively spliced forms, one of which contains a full FN-like repeat, remains to be determined by other methods than Northern blot analysis. It  
25 is noteworthy in this context that no evidence for alternative splicing was found by restriction analysis of the six independently isolated clones (not shown). Alternative splicing of the fifth FN-like domain was observed for Nr-CAM/BRAVO, where cDNA isoforms were isolated lacking the fifth FN-like repeat (Grumet et al., 1991; Kayyem et al., 1992.) The absence of the fifth FN-  
30 like domain in chicken neurofascin (Volkmer et al., 1992) is most probably also

due to alternative splicing. since its rat homolog, the ankyrin-binding glycoprotein (ABGP) (Davis et al., 1993), contains a fifth FN-like domain. Thus, CHL1 adds a new structural feature to the L1 family: only four and one-half FN-like repeats are expressed (Figure 19).

- 5 Another structural feature of CHL1 is the presence of an RGD sequence (amino acids 185-187) in the second Ig-like domain (Figure 18. This tripeptide has originally been identified as a cell attachment site within the tenth type III domain of fibronectin (Pierschbacher and Rusolahti, 1984) and contributes to integrin binding (for review see Rusolahti and Pierschbacher, 1987). Three dimensional  
10 structure analysis of FN-like repeats showed that the RGD motif is localized between the  $\beta$ -strands F and G (Main et al., 1992). This motif is also found in other members of the L1 family. In the third FN-like repeats of chicken Ng-CAM (Burgoon et al., 1991) and the species homologs chicken neurofascin and rat ABGP, the RGD sequence is found at the same position, between the  $\beta$ -strands F  
15 and G. RGD motifs are also found in L1 (two in L1 mouse and rat (NILE), and one in human L1 (Moos et al., 1988; Hlavin and Lemmon, 1991; Prince et al., 1991). All L1 RGD sequences are found in the sixth Ig-like domain, but in a different amino acid environment than RGDs in the FN-like modules of fibronectin. As in L1, the tripeptide in CHL1 is localized on the  $\beta$ -strand E of the second Ig-  
20 like domain. Whether the RGD sequences in these proteins are functionally active is currently not known. It is noteworthy in this context that neurite extension induced by TAG-1 (Furley et al., 1990), a member of the F3/F11 family (Brümmendorf and Rathjen, 1993, 1994) that contains a RGD motif in the second FN-like domain depends on  $\beta_1$  integrin and L1 (Felsenfeld et al., 1994). This  
25 observation raises the possibility of a direct physical interaction between the second FN-like repeat of TAG-1 and  $\beta_1$  integrin.

CHL1 also contains a DGEA sequence (amino acids 555-558) in the  $\beta$ -strand C of the sixth Ig-like domain (Figure 18). This sequence is not found in other members

of the L1 family. The DGEA sequence has also been implicated in  $\alpha_2\beta_1$  integrin recognition of type I collagen containing this motif (Staatz et al., 1991).

**Structural similarity of CHL1 with other recognition molecules of the Ig superfamily**

- 5 A comparison of the amino acid sequence of CHL1 with the translated EMBL gene sequence database showed that CHL1 is 87.2% identical to a 109 amino acid long stretch and 79.6 identical to a 93 amino acid long stretch previously identified in human brain (accession number HS2431 and HSXT02610 (Adams et al., 1992, 1993)). Thus, there appears to be a highly conserved CHL1 molecule in human.
- 10 The sequences of mouse, human, and rat L1/NILE, chicken Ng-CAM, chicken Nr-CAM, zebrafish L1.1 (Tongiorgi et al., 1995), chicken neurofascin/rat ABGP, *Drosophila* neuroglian (Bieber et al., 1989), mouse F3/chicken F1/human CNTN1 (Gennarini et al., 1989; Ranscht, 1988; Brummendorf et al., 1989; Berglund and Ranscht, 1994), rat TAG-1/ chicken axonin-1/ human TAX-1 (Furley et al., 1990; Hasler et al., 1993; Tsiotra et al., 1993), and rat BIG-1/ mouse PANG (Yoshihara et al., 1994; Connelly et al., 1994) taken from the translated EMBL gene sequence database were compared with CHL1. The comparison is displayed in Table 1, below.
- 15

TABLE 1: Comparison of sequence similarities of the extracellular parts of CHL1 and other L1 related molecules

|                | CHL1<br>(m) | L1<br>(m) | ngCAM<br>(c) | NrCAM<br>(c) | neurofascin<br>(c) | L1.1<br>(z) | neuroglian<br>(d) | F3<br>(m) | TAG<br>-1 | BIG-1<br>(r) |
|----------------|-------------|-----------|--------------|--------------|--------------------|-------------|-------------------|-----------|-----------|--------------|
| L1 (m)         | 37          |           |              |              |                    |             |                   |           |           |              |
| NgCAM (c)      | 39          | 37        |              |              |                    |             |                   |           |           |              |
| NrCAM (c)      | 33          | 43        | 35           |              |                    |             |                   |           |           |              |
| neurofascin(c) | 36          | 36        | 47           | 33           |                    |             |                   |           |           |              |
| L1.1 (zf)      | 28          | 28        | 30           | 26           | 30                 |             |                   |           |           |              |
| neuroglian (d) | 33          | 36        | 36           | 34           | 34                 | 28          |                   |           |           |              |
| F3 (m)         | 27          | 28        | 27           | 26           | 27                 | 26          | 24                |           |           |              |
| TAG-1 (r)      | 27          | 27        | 26           | 25           | 25                 | 25          | 24                | 51        |           |              |
| BIG-1 (r)      | 27          | 29        | 27           | 27           | 25                 | 27          | 24                | 44        | 43        |              |
| DCC (h)        | 14          | 14        | 14           | 15           | 14                 | 16          | 14                | 16        | 15        | 15           |
| Ax1 (m)        | 11          | 12        | 11           | 12           | 12                 | 11          | 11                | 13        | 12        | 11           |
| HLAR (h)       | 15          | 15        | 13           | 14           | 13                 | 14          | 11                | 13        | 14        | 14           |
| NCAM (m)       | 16          | 15        | 17           | 16           | 15                 | 16          | 15                | 15        | 15        | 15           |

The extracellular regions of the members of the L1 family were compared with each other and with other and with other neural cell adhesion molecules of the Ig superfamily. Values indicate the percentage of amino acid identity after alignment according to Hein (1990). The species are indicated in brackets: c = chicken, d = Drosophila, h = human, m = mouse, r = rat, and zf = zebrafish.

TABLE 2: Comparison of sequence similarities of the intracellular parts of L1 related molecules

|                  | CHL1(m) | L1(m,r,h) | NrCAM(m) | NrCAM(c) | NrCAM(c) | ABOP(r) | neurofascin(m) | neurofascin(c) | neuroglian(r) | L1.1 (sf) | L1.2 (sf) |
|------------------|---------|-----------|----------|----------|----------|---------|----------------|----------------|---------------|-----------|-----------|
| L1 (m, r, h)     | 57      |           |          |          |          |         |                |                |               |           |           |
| NrCAM (m)        | 64      | 55        |          |          |          |         |                |                |               |           |           |
| NrCAM (c)        | 62      | 56        | 99       |          |          |         |                |                |               |           |           |
| NrCAM (c)        | 43      | 61        | 48       | 43       |          |         |                |                |               |           |           |
| ABOP (r)         | 59      | 54        | 64       | 64       | 38       |         |                |                |               |           |           |
| neurofascin (m)  | 62      | 56        | 64       | 65       | 43       | 100     |                |                |               |           |           |
| neurofascin (c)  | 54      | 58        | 58       | 60       | 40       | 87      | 86             |                |               |           |           |
| neuroglian (r)   | 39      | 34        | 41       | 40       | 34       | 40      | 43             | 37             |               |           |           |
| L1.1 (sf)        | 43      | 61        | 46       | 45       | 50       | 45      | 48             | 47             | 36            |           |           |
| L1.2 (sf)        | 36      | 54        | 39       | 38       | 37       | 37      | 40             | 39             | 22            | 44        |           |
| DOC (h)          | 14      | 12        | 15       | 13       | 18       | 12      | 13             | 12             | 11            | 12        | 9         |
| fascilin III (d) | 21      | 17        | 20       | 18       | 19       | 16      | 20             | 17             | 15            | 17        | 15        |
| MAO p72 (r)      | 8       | 10        | 11       | 11       | 10       | 10      | 10             | 11             | 11            | 10        | 8         |
| NCAM180 (m)      | 7       | 7         | 7        | 7        | 13       | 5       | 5              | 5              | 5             | 13        | 9         |
| Po (m)           | 11      | 6         | 12       | 13       | 6        | 15      | 15             | 15             | 28            | 7         | 3         |

The intracellular regions of the members of the L1 family (including species homologs) were compared with each other and with other neural cell adhesion molecules of the Ig superfamily. Values indicate the percentage of amino acid identity after alignment according to Hein (1990). The species are indicated in brackets: c = chicken, d = Drosophila, h = human, m = mouse, r = rat, and sf = zebrafish. Mouse, rat, and human L1 are identical. Mouse NrCAM and neurofascin are partial sequences of 91 and 86 amino acid residues, respectively.

TABLE 3: Structural relationships between members of the LI family and other members of the Ig superfamily containing C2 domains

[illegible]



TABLE 3: Structural relationships between members of the L1 family and other members of the Ig superfamily containing C2 domains (Continued)

| CAMs              | IgG-FN1 | FN1 | FN1-FN2 | FN2 | FN2-FN3 | FN3 | FN3-FN4 | FN4 | Similarity Index |
|-------------------|---------|-----|---------|-----|---------|-----|---------|-----|------------------|
| L1                | 44      | 50  | 50      | 49  | 49      | 58  | 47      | 51  | 84%              |
| CHLI              | 41      | 7   | 7       | 49  | 49      | 58  | 48      | 53  | 84%              |
| NCAM              | 40      | 50  | 50      | 57* | 49      | 77* | 7       | 7   | 74%              |
| NCAM              | 51*     | 50  | 50      | 50  | 49      | 58  | 48      | 52  | 95%              |
| ABCP              | 56*     | 50  | 50      | 50  | 49      | 58  | 48      | 51  | 95%              |
| L1.1              | 41      | 50  | 50      | 50  | 49      | 62  | 47      | 52  | 74%              |
| neuroglin         | 40      | 50  | 50      | 52  | 51      | 53  | 7       | 54  | 48%              |
| TAG-1             | 41      | 53  | 50      | 53  | 49      | 51  | 48      | 7   | 74%              |
| BIO-1             | 41      | 53  | 50      | 53  | 49      | 52  | 48      | 47  | 84%              |
| F3                | 41      | 53  | 50      | 53  | 49      | 48* | 48      | 47  | 43%              |
| Average           | 41      | 51  | 50      | 51  | 49      | 56  | 48      | 51  |                  |
| SD                | 1.2     | 1.4 | 0.0     | 1.7 | 0.6     | 3.8 | 0.5     | 2.6 |                  |
| DCC               |         | 49  | 50      | 46  | 49      | 51  | 51      | 51  | 50%              |
| HLAR              |         | 46  | 49      | 50  | 48      | 46  | 49      | 53  | 42%              |
| rbc               |         | 49  | 48      | 49  |         |     |         |     | 33%              |
| NCAM              |         | 35  | 56      | 41  |         |     |         |     | 25%              |
| MAO               |         |     |         |     |         |     |         |     | 11%              |
| actinomyosin      |         |     |         |     |         |     |         |     | 13%              |
| fibrinectin (III) |         | 45  | 42      | 46  | 48      | 44  | 47      | 44  | 14%              |

The table shows the number of residues between conserved amino acids within the Ig-like domains (cysteines involved in the S-S bridges: Ig1, Ig2, Ig3, Ig4, Ig5, and Ig6), FN-like repeats (tryptophan of the second  $\beta$ -strand and tyrosine/phenylalanine of the sixth  $\beta$ -strand: FN1, FN2, FN3, and FN6), and between these domains (1-2, 2-3, 3-4, 4-5, and 5-6; FN1-FN2, FN2-FN3, and FN3-FN4). The distance between the conserved amino acids of the second cysteine of the sixth Ig-like domain and the first tryptophan of the first FN-like repeat (Ig6-FN1) reflects the distance between the Ig-like domains and FN-like repeats. The average and standard deviation (SD) of the individual distances for the L1 related molecules are indicated, DCC, HLAR, rbc, NCAM, MAG, neuromusculin, and fibrinectin (III) are given as a control. Similarity Index is given at the right margin. ? = no conserved amino acid, \* = not used for average and SD calculations.

CHL1 is most similar to chicken Ng-CAM (37% amino acid identity in the extracellular domain. Table 1) and mouse Nr-CAM (64% amino acid identity in the intracellular domain. Table 2). However, the degree of identity, particularly in the extracellular part, is not sufficient to consider these proteins as species homologs.

5 Recently, a partial cDNA clone of mouse Nr-CAM (Moscoso and Sanes, 1995) was identified. Mouse Nr-CAM is nearly identical (99%) to chicken NR-CAM (see Table 2). Therefore, CHL1 is not likely the Nr-CAM homolog in the mouse. CHL1 is the fourth member of the L1 family in the mouse with L1, Nr-CAM, neurofascin (Moscoso and Sanes, 1995), and CHL1, with a highly conserved

10 species homolog in human (Adams et al., 1992, 1993).

Considering the similarity of the intracellular sequences of chicken and mouse Nr-CAM and of chicken and mouse neurofascin (99% and 87%, respectively, Table 2), it is highly unlikely that mouse L1 and chicken Ng-CAM are species homologs, since they show only 01% sequence identity in the intracellular domain (Table 2).

15 Rather, the existence of Ng-CAM as the fifth member of the L1 family in the mouse with a highly conserved intracellular domain is to be expected. Interestingly, mouse L1 upon heterophilic interaction with chicken Ng-CAM promotes neurite outgrowth (Lemmon et al., 1989), suggesting that members of the L1 family may interact with each other.

20 Besides the similarities in the overall structure of L1 family members (Table 1 and Table 3), the most highly conserved regions can be identified in the cytoplasmic domain (Figure 20). This striking homology is evident for members of the L1 family in all species so far identified which contain an intracellular domain: L1, CHL1, Nr-CAM, Ng-CAM, neurofascin, neuroglian, and zebrafish L1.1 and also

25 for the partial sequences of zebrafish L1.2 (Tongiorgi et al., 1995) and mouse Nr-CAM and neurofascin (Moscoso and Sanes, 1995) (Table 2). Within this region two stretches, one located close to and partially within the plasma membrane-spanning segment and the other at its C-terminal end (I, III in Figure 20), are nearly identical. Another amino acid stretch conserved in L1, Ng-CAM, Nr-CAM,

30 neurofascin, L1.1 and L1.2 but not in CHL1 (Table 2) contains a RSLE motif (II in Figure 3) that originates by alternative splicing and is expressed only in neurons

(Grumet et al., 1991; Miura et al., 1991; Volkmer et al., 1992). Since the intracellular region is most highly conserved between these proteins, all members of the L1 family may use the same signal transduction pathway to activate neurite extension. It has been demonstrated that the cytoplasmic domains of ABGP, L1, and Nr-CAM can interact with ankyrin linking cell recognition to the cytoskeletal scaffold (Davis et al., 1993, Davis and Bennett, 1994).

#### **EXAMPLE 24**

##### **Identification of structural requirements in the extracellular domain to classify members of the L1 family**

- 10 To further study the general criteria for membership in the L1 family, we investigated the position of highly conserved amino acids in the Ig-like domains (cysteines which refer to the S-S bridges) and FN-like repeats (tryptophan, tyrosine/phenylalanine) for several members of the Ig superfamily (Table 3). Molecules containing six Ig-like domains and at least four FN-like repeats (L1 family and the GPI linked F3/F11 subgroup (Brümmendorf and Rathjen, 1993))
- 15 reveal a very constant number of amino acids separating these conserved amino acids. Five different distance parameters were considered:
- 1) the number of amino acids separating the conserved cysteines which form the cysteine (S-S) bridges of each Ig-like domain (Table 3, columns Ig1, Ig2, Ig4, Ig5,
  - 20 and Ig6);
  - 2) the number of amino acids between the second cysteine of one Ig-like domain and the first cysteine of the next Ig-like domain reflecting the distance between two adjacent Ig-like domains (Table 3, columns 1-2, 2-3, 3-4, 4-5, and 5-6):
  - 3) the number of amino acids between the last conserved cysteine of the sixth Ig-
  - 25 like domain and the conserved tryptophan of the first FN-like repeat reflecting the distance between the Ig-like domain-module and the FN-like repeat-module (Table 3, columns IgG-FN1):
  - 4) the number of amino acids between the conserved tryptophan, tyrosine/phenylalanine of each individual FN-like repeat (Table 3, columns FN1,
  - 30 FN2, FN3, and FN4):
  - 5) the number of amino acids between the tyrosine/phenylalanine of one FN-like repeat and the tryptophan of the next FN-like repeat reflecting the distance between

two adjacent FN-like repeats (Table 3. columns FN1-FN2, FN2-FN3, and FN3-FN4).

To obtain highly stringent conditions for comparison of the L1-like molecules, we did not consider a few values clearly deviating from the average, most probably due to alternative splicing, for the calculation of the average distances and standard deviations (neuroglian: Ig2, 2-3: Nr-CAM and ABGP: Ig6-FN1; F3: Ig4, FN3; Ng-CAM; FN2, FN3 (Table 3, marked with an \*)). Whereas the number of amino acids between the S-S bridges for the first and sixth Ig-like domain (Ig1; standard deviation (SD) = 3; Ig6: SD = 4) and the number of amino acids between the conserved tryptophan, tyrosine/phenylalanine of FN-like repeats three and four (FN3: SD = 4; FN4: SD = 3) are slightly variable, all other distance parameters remain remarkably constant for the different molecules (FN1-FN2: SD = 0; FN2 and 2-3: SD = 2 (Table 3)). Based on these criteria we calculated a similarity index (see Material and Methods) for several Ig-like molecules in relation to the average values listed in Table 3. For L1, CHL1, Ng-CAM, Nr-Cam, ABGP, L1.1, TAG-1 and BIG-1 a similarity index of 74-95% was obtained (Table 3). For *Drosophila* neuroglian a slightly lower value was determined (66%), most probably reflecting the evolutionary distance between vertebrates and insects. F3 and its species homologs are less conserved, particularly in their Ig-like domains, but still show a similarity index of 63%. However, some conserved amino acid stretches underlying the strongly conserved colinearity (e.g. FxVxAxNxxG(8x)S(4x)TxxAxPxxxP at the end of the first FN-like repeat or NxxGxGPxS between the last two  $\beta$ -strands of the third FN-like repeat (not shown) support the notion that F3 belongs to the L1 family. Interestingly, the number of amino acids between adjacent domains (Ig-like domains or FN-like repeats) is even more conserved among these molecules, indicating that the distance between the individual domains is an important structural feature, i.e. critical for functioning of neural recognition molecules (Table 3. columns 1-2, 2-3, 3-4, 4-5, 5-6, FN1-FN2, FN2-FN3, and FN3-FN4). Thus, this high conservation of the order (colinearity) and spacing may be used to define more generally the extracellular domain of the L1 family members. With the criteria just defined, these contain a module of six

Ig-like domains at the N-terminus followed by four FN-like repeats. We would like to call this structural feature the L1 family cassette.

Thus, all members of the L1 family share the characteristic features of the L1 family cassette and, additionally, highly conserved amino acids. These results suggest that these molecules derive from a common ancestral L1-like molecule containing the L1 family cassette, which might have spread its function potential via gene duplication to accommodate the evolving demands for diversifying cell interactions in more complex nervous systems. The general L1 family may thus be subdivided into the "classical" L1 family members (L1, CHL1, Ng-CAM, Nr-CAM, neurofascin, neuroglian, L1.1) which contain a variable fifth FN-like repeat, a transmembrane domain, and a highly conserved intracellular domain, and the F3/F11 subgroup (F3/F11/CNTN1, BIG-1/PANG, and TAG-1/Axonin-1/TAX-1), the common feature of which is the linkage by GPI to the membrane and for which a variable fifth FN-like repeat has so far not been identified. The extracellular domains of both subgroups contain the L1 family cassette.

Other members of the Ig-superfamily, e.g. N-CAM (Cunningham et al., 1987, Barthels et al., 1987). MAG (Arquint et al., 1987; Lai et al., 1987; Salzor et al., 1987), neuromusculin (Kanla et al., 1993), and rse (Mark et al., 1994) or fibronectin (Kornblihtt et al., 1985) which contain Ig-like domains and/or FN-like repeats, show clearly distinct distance parameters, indicating that they are much less related to each other and to the members of the L1 family (Table 3). Interestingly, the human leukocyte common antigen-related gene (HLAR) (Streull et al., 1988) and the tumor suppressor gene product (DCC) deleted in colorectal cancer (Fearon et al., 1990) are closely related to the L1 family according to the distance parameters (42% and 50% similarity index, respectively). Inspection of conserved amino acids reveals that DCC is indeed related more closely to the L1 family than to N-CAM as previously suggested by Fearon et al. (1990) and Pierceall et al. (1994), although it seems to have lost the fifth and sixth Ig-like domain. Recent studies show that DCC is expressed predominantly in brain and that neurite outgrowth of rat PC12 cells is stimulated on a substrate of DCC-transfected fibroblasts expressing the protein on its cell surface (Pierceall et al., 1994).

Although HLAR also shows a relatively high similarity index its relationship to the L1 family is not so obvious.

### EXAMPLE 25

#### **Tissue distribution of CHL1 mRNA and Protein**

To investigate whether CHL1 shares the predominant expression in the nervous system with other members of the L1 family, we analyzed the expression of CHL1 in various tissues at the mRNA and protein levels. In Northern blot analysis the CHL1 riboprobe hybridized with a predominant mRNA band of approximately 8 kb (Figure 21) which is significantly larger than the size of the mRNA detected with L1 probes (approximately 6 kb) (Tacke et al., 1987). The smaller and weaker RNA band (Figure 21a, lane 3) is most probably due to the cross-hybridization with ribosomal RNA. The 8 kb RNA was detected in cerebellum, brain minus cerebella, and spinal cord but not in dorsal root ganglia (DRG) (Figure 21a). In contrast, the L1 riboprobe showed a strong signal with RNA from DRG (Figure 21a). CHL1 mRNA was also detectable in nine-day-old rat cerebellum and six-day-old rat spinal cord but not in rat PC12 cells maintained with and without NFG or in COS-1 cells (Figure 21b). In all other tissues analyzed (thymus, lung, liver, intestine, spleen, and kidney) no signal was detectable (Figure 21a).

To identify the CHL1 protein, antibodies to a bacterially expressed fragment of the CHL1 protein were generated. Excluding regions of high homology to other known L1 family members, e.g. transmembrane spanning or intracellular regions, a 1.7 kD cDNA fragment representing part of the sixth Ig-like domain and the four FN-like repeats (Figures 18 and 19b) was cloned into the pET expression vector. Expression of the resulting protein fragment led to a 70 kD band detected by Coomassie blue staining after SDS-PAGE which was purified by anion exchange chromatography. Western blot analysis (Figure 22a) and ELISA (not shown) showed that the antisera from two rabbits reacted with the CHL1 protein fragment but not with purified L1, N-CAM, or MAG. Some reaction with bacterial proteins copurified with the CHL1 peptide or degradation products of the CHL1 fragment was observed (Figure 22a, lane 4).

To further examine the specificity of the antibodies and to determine whether they recognize the native cell surface expressed CHL1, transiently transfected COS-1 cells were examined with the antibodies. Immunocytochemistry revealed cell

surface expression of CHL1 on CHL1-transfected cells, but not on cells mock-transfected with the vector (Figure 23). These results also demonstrate that the putative signal sequence is functional and that the open reading frame is correct.

Although the first CHL1 cDNA clone was isolated from an expression library by  
5 screening with immunoaffinity purified polyclonal antibodies against brain derived L1, reaction of a different preparation of L1 antibodies against the recombinantly expressed Ig-like domains of L1 with CHL1-transfected cells was not observed (not shown). Also CHL1 antibodies directed against the extracellular part of the molecule (Figure 19) did not react with L1-transfected cells (not shown). It is  
10 therefore likely that the L1 polyclonal antibodies used for screening the expression library were reactive with the C-terminal, intracellular part of CHL1 which is most homologous between CHL1 and L1.

The CHL1 antisera were used to identify immunoreactive proteins in several tissues (brain, liver, lung, kidney, and intestine from nine-day old mice. Figure 23b).  
15 Crude membrane fractions, soluble and insoluble in 0.5 % Triton X-100, were analyzed by Western blotting. Polyclonal antibodies against L1 were used as a control. The CHL1 antibodies recognized three distinct bands of 185, 165, and 125 kD in the insoluble and soluble fractions of brain membranes. The 185 kD band was only weakly detectable in the soluble fraction and the 125 kD band was less  
20 prominent in the insoluble fraction (Figure 23b, lane 1 and 2), indicating that the 185 kD band is probably the membrane bound form of CHL1, whereas the 125 and 165 kD forms are probably proteolytically cleaved fragments. A similar pattern of immunoreactive bands was observed after Western blot analysis of CHL1 transfected COS cells and total brain tissue (not shown). A similar pattern of  
25 bands has been observed for L1 (Faissner et al., 1985; Sadoul et al., 1988), Ng-CAM (Grumet et al., 1984), and NrCAM (Kayyem et al., 1992). However, a dibasic consensus sequence for proteolytic cleavage in the third FN-like domain (L1: "SKR": Ng-CAM: "SRR": Nr-CAM: "SRR": Nr-CAM: "SRRSKR") is not present in CHL1. Like the other members of the L1 family, CHL1 was found to  
30 be expressed only at later stages of development. It was not detectable in brain before embryonic day 15 by Western blot analysis (not shown). A 50 kD



immunoreactive band was detected in the detergent soluble fraction of whole liver tissue. This band is most probably due to some cross-reactivity of the CHL1 antibodies with a CHL1-related protein, since no CHL1-specific mRNA was seen in liver by Northern blot analysis (Figure 22a). No CHL1 immunoreactivity could be  
5 detected in the other tissues that were tested (Figure 23b).

In the CNS, members of the L1 family are predominantly expressed by neurons. Therefore, we were interested if CHL1 shares this pattern of expression with L1. *In situ* hybridization experiments were performed to identify the cells synthesizing CHL1 and L1 in the retina, optic nerve, and cerebellar cortex of young postnatal  
10 mice. In the retina of 7-day-old mice, L1 (Fig. 24a) and CHL1 mRNA (Fig. 24b) are expressed by ganglion cells. L1 transcripts were additionally detectable in amacrine and horizontal cells located in the inner nuclear layer (Fig. 24a). CHL1 mRNA, in contrast, was only occasionally detectable in a few cells located at the inner (i.e. vitread) margin of the inner nuclear layer (Fig. 24b). Glial cells in the  
15 optic nerve did not contain detectable levels of L1 transcripts (Fig. 24a). In striking contrast, CHL1 mRNA was strongly expressed by glial cells located in proximal (i.e. retina-near) regions of the optic nerve (Fig. 24b) and low levels of CHL1 expression were visible in glial cells located in more distal regions of the nerve (Fig. 24b).

20 In the cerebellar cortex of two-week-old mice, L1 mRNA was detectable in stellate and basket cells located in the molecular layer and in Golgi and granule cells located in the internal granular layer (Fig. 24d). The same cells types were labeled when sections were hybridized with the CHL1 antisense cRNA probe (Fig. 24e), with the exception that CHL1 transcripts were hardly detectable in cells located in  
25 the inner part of the molecular layer (compare Fig. 24d and e). As a negative control, sections were hybridized with the corresponding sense cRNA probes and no labeling of cells was detectable (for a retina and optic nerve hybridized with a CHL1 sense cRNA probe, see Fig. 24c). In order to address whether glial cells express CHL1 *in vitro*, cultures of purified astrocytes or oligodendrocytes were  
30 prepared from the forebrain of young postnatal mice or rats. The same polyclonal CHL1 antibodies which specifically detected CHL1 at the cell surface of

transfected COS-1 cells were used. Astrocytes and oligodendrocytes were identified with antibodies to GFAP or with antibodies to the O1 antigen, respectively. Astrocyte cultures contained some cells which were double-labeled by polyclonal CHL1 (Fig. 25a, d) and monoclonal GFAP (Fig. 25b, e) antibodies.

- 5 Analysis of oligodendrocyte cultures, however, revealed no co-localization of CHL1 and the O1 antigen, indicating that mature oligodendrocytes *in vitro* do not express detectable levels of CHL1. The combined observations indicate that CHL1 and L1 show overlapping but also distinct patterns of expression. Most strikingly CHL1, but not L1, is expressed by certain glial cells of the nervous system *in vivo*,  
10 suggesting that different members of the L1 family perform different functions.

**Analysis of glycosylation and detection of the HNK-1 carbohydrate by the CHL1 glycoprotein**

- Since the observed molecular weight of CHL1 (185 kD) is considerably larger than the calculated molecular mass (134.9 kD), the carbohydrate contribution to the  
15 molecular mass and the type of carbohydrate modification was analyzed. The detergent soluble and insoluble fractions from crude brain membranes of seven-day-old mice were subjected to enzymatic deglycosylation. After N-glycosidase F (PNGaseF) treatment the molecular mass of all CHL1 immunoreactive proteins was reduced (Figure 26): The 185 kD band shifted to 150 kD, the 165 kD band to 135  
20 kD, and the 125 kD band to 110 kD. Treatment with O-glycosidase, an enzyme known to cleave serine/threonine linked galactosyl  $\beta(1-3)$ N-acetylgalactosaminyl disaccharides (Glasgow et al., 1977) resulted in a slightly increased mobility: The 185 and 165 kD bands shifted to about 180 and 160 kD, respectively, whereas the 125 kD band did not shift. These observations indicate that most of the  
25 carbohydrate molecular mass is due to N-linked carbohydrates. Treatment with both enzymes together (Figure 26) led to a larger shift than seen with treatment with individual enzymes from 185 to 145 kD, suggesting that not all glycosylation sites, most probably the O-glycosylation sites were cleaved by O-glycosidase alone. The results show that CHL1 contains approximately 30 % of its molecular mass as  
30 N-glycosidically linked carbohydrates.

Several neural cell adhesion molecules carry the HNK-1 carbohydrate, such as L1 (Kruse et al., 1984), TAG-1 (Dodd et al., 1988), Nr-CAM (Grumet et al., 1991), F3 (Gennarini et al., 1989), N-CAM (Kruse et al., 1984), the myelin associated glycoprotein MAG (McGarry et al., 1983; Kruse et al., 1984), and P<sub>0</sub> (Bollenson and Schachner, 1987). Therefore, we analyzed whether CHL1 carries the HNK-1 carbohydrate. CHL1 was immunoprecipitated from detergent lysates of whole brain tissue from nine-day-old mice with CHL1 antibodies. As control, L1 was similarly immunoprecipitated with polyclonal antibodies from the same brain extract. Western blot analysis with monoclonal antibody 412 directed against the HNK-1 carbohydrate epitope showed that both immunoprecipitates contained bands which were recognized by the monoclonal antibody 412 at molecular masses expected for CHL1 (Figure 27) or L1 (not shown). Since the HNK-1 carbohydrate is involved in cell-to-cell adhesion and binds to laminin (Keilhauer et al., 1985; Künemund et al., 1988; Hall et al., 1993, 1995), CHL1, like the other members of the L1 family, may interact with laminin via the HNK-1 carbohydrate.

## CONCLUSIONS

The above experiments has added CHL1 as another member of the L1 family of neural recognition molecules found in such diverse species as human, rat, mouse, chicken, zebrafish, and *Drosophila*, thus constituting a phylogenetically conserved family of molecules all of which are expressed late in development at the onset of axogenesis by neurons and subsets of neurons. The fact that many L1 related molecules exist points to nature's requirement for structurally similar, but functionally most likely distinct neurite outgrowth promoting molecules, and to the evolution of the L1 family as a group of molecules that may determine the fine-tuning of axonal pathfinding.

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- While the invention has been described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Numerous variations of such details can be
- 25 implied as will be appreciated by those skilled in the art.



WHAT IS CLAIMED IS:

- 1     1.     A method for promoting neural growth *in vivo* in the central nervous system  
2     of a mammal comprising administering to said mammal a neural growth promoting  
3     amount of an agent, said agent comprising a neural cell adhesion molecule, which  
4     molecule is capable of overcoming inhibitory molecular cues found on glial cells  
5     and myelin and promoting said neural growth, active fragments thereof, cognates  
6     thereof, congeners thereof, mimics thereof, antagonists thereof, antibodies thereto,  
7     analogs thereof, secreting cells thereof and soluble molecules thereof.
- 1     2.     The method of Claim 1 wherein said agent is derived from members of the  
2     immunoglobulin superfamily that mediate  $Ca^{2+}$ -independent neuronal cell adhesion.
- 1     3.     The method of Claim 1 wherein said agent is derived from molecules that  
2     contain structural motifs similar to fibronectin type III homologous repeats and  
3     immunoglobulin-like domains.
- 1     4.     The method of Claim 3 wherein said structural motifs are structurally  
2     similar to fibronectin type III homologous repeats 1-2, and immunoglobulin-like  
3     domains I-II, III-IV, and V-VI.
- 1     5.     The method of Claim 2 wherein said agent is selected from the group  
2     consisting of L1, N-CAM and myelin-associated glycoprotein.
- 1     6.     The method of Claim 2 wherein said agent is selected from the group  
2     consisting of laminin, fibronectin, N-cadherin, BSP-2 (mouse N-CAM), D-2, 224-  
3     1A6-A1, L1-CAM, NILE, Nr-CAM, TAG-1 (axonin-1), Ng-CAM and F3/F11.
- 1     7.     A recombinant DNA molecule for use in the method of any of Claim 1-6,  
2     comprising the agent, or an active fragment, cognate, congener, mimic or analog  
3     thereof, associated with an expression control sequence.
- 1     8.     A vector comprising the recombinant DNA molecule of Claim 7.

- 1 9. A transformed host containing the vector of Claim 8.
- 1 10. An antibody raised to the agent of any of Claim 1-6.
- 1 11. The antibody of Claim 10 comprising a polyclonal antibody.
- 1 12. The antibody of Claim 10 comprising a monoclonal antibody.
- 1 13. A method for modulating neural growth in the central nervous system of a  
2 mammal comprising administering to said mammal a neural growth-modulating  
3 amount of the antibody of Claim 10, or the active fragments, cognates, congeners,  
4 analogs, mimics, secreting cells or soluble molecules thereof.
- 1 14. A pharmaceutical composition for the modulation of neural growth in the  
2 central nervous system of a mammal, comprising a therapeutically effective amount  
3 of the agent of Claim 1, agonists thereof, antagonists thereto, fragments thereof,  
4 cognates thereof, congeners thereof, mimics, analogs, secreting cells or soluble  
5 molecules thereof, and a pharmaceutically acceptable carrier.
- 1 15. A transgenic mammal comprising glial cells which express an exogenous  
2 neural adhesion molecule.
- 1 16. The transgenic mammal of Claim 15, wherein the glial cells are astrocytes.
- 1 17. The transgenic mammal of Claim 15, wherein the neural adhesion molecule  
2 is L1.
- 1 18. A cell culture comprising the glial cells of the transgenic mammal of one of  
1 Claims 15-17.
- 1 19. A cell culture system comprising tissue from the central nervous system of  
2 the transgenic mammal of one of Claims 15-17.

- 1 20. A method for enhancing neuronal outgrowth of CNS neurons, comprising  
2 culturing said neurons on the cell culture system of Claim 18.
- 1 21. A method for enhancing neuronal outgrowth of CNS neurons, comprising  
2 culturing said neurons on the cell culture system of Claim 19.
- 1 22. A method for enhancing memory, comprising administering to the brain of  
2 a mammal in need of such enhancement, an amount of the cells of the cell culture  
3 system of Claim 18 effective to enhance the memory of the mammal.
- 1 23. A method for enhancing memory, comprising administering to the brain of  
2 a mammal in need of such enhancement, an amount of the cells of the cell culture  
3 system of Claim 19 effective to enhance the memory of the mammal.
- 1 24. A method for enhancing memory, comprising delivering to the glial cells of  
2 the brain of a mammal in need of such enhancement, a vector which allows for the  
3 expression of a neural adhesion molecule in said glial cells.
- 1 25. The method of Claim 24, wherein the neural adhesion molecule is L1.
- 1 26. A method for increasing synaptic efficacy in the CNS of a mammal in need  
2 of such an increase, comprising administering to the brain of the mammal, an  
3 amount of the cells of the cell culture system of Claim 18 effective to increase  
4 synaptic efficacy in the brain of the mammal.
- 1 27. A method for increasing synaptic efficacy in the CNS of a mammal in need  
2 of such an increase, comprising administering to the brain of the mammal, an  
3 amount of the cells of the cell culture system of Claim 19 effective to increase  
4 synaptic efficacy in the brain of the mammal.
- 1 28. A method for increasing synaptic efficacy in the CNS of a mammal in need  
2 of such an increase, comprising delivering to the glial cells of the brain of a

3 mammal in need of such enhancement, a vector which allows for the expression of  
4 a neural adhesion molecule in said glial cells.

1 29. The method of Claim 26, wherein the increase in synaptic efficacy is  
2 demonstrated by the stabilization of long term potentiation.

1 30. The method of Claim 27, wherein the increase in synaptic efficacy is  
2 demonstrated by the stabilization of long term potentiation.

1 31. The method of Claim 28, wherein the increase in synaptic efficacy is  
2 demonstrated by the stabilization of long term potentiation.

1 32. A method of testing the ability of a drug or other entity to modulate the  
2 activity of a neural adhesion molecule which comprises:  
3 a. adding CNS neurons to the cell culture system of Claim 18;  
4 b. adding the drug under test to the cell culture system;  
5 c. measuring the neuronal outgrowth of the CNS neurons; and  
6 d. correlating a difference in the level of neuronal outgrowth of cells in  
7 the presence of the drug relative to a control culture to which no drug is added to  
8 the ability of the drug to modulate the activity of the neural adhesion molecule.

1 33. A method of testing the ability of a drug or other entity to modulate the  
2 activity of a neural adhesion molecule which comprises:  
3 a. adding CNS neurons to the cell culture system of Claim 19;  
4 b. adding the drug under test to the cell culture system;  
5 c. measuring the neuronal outgrowth of the CNS neurons; and  
6 d. correlating a difference in the level of neuronal outgrowth of cells in  
7 the presence of the drug relative to a control culture to which no drug is added to  
8 the ability of the drug to modulate the activity of the neural adhesion molecule.

1 34. The method of Claim 32, wherein the neural adhesion molecule is L1.

1 35. The method of Claim 33, wherein the neural adhesion molecule is L1.

1     36.     An assay system for screening drugs and other agents for ability to  
2     modulate the production of a neural adhesion molecule, comprising:  
3             a.     culturing the cell culture system of Claim 18 inoculated with a drug  
4     or agent;  
5             b.     adding CNS neurons to the cell culture system of step a); and  
6             c.     examining neuronal outgrowth to determine the effect of the drug  
7     thereon.

1     37.     An assay system for screening drugs and other agents for ability to  
2     modulate the production of a neural adhesion molecule, comprising:  
3             a.     culturing the cell culture system of Claim 18 inoculated with a drug  
4     or agent;  
5             b.     adding CNS neurons to the cell culture system of step a); and  
6             c.     examining neuronal outgrowth to determine the effect of the drug  
7     thereon.

1     38.     The assay system of Claim 36, wherein the neural adhesion molecule is  
2     selected from the group consisting of laminin, fibronectin, N-cadherin, BSP-2  
3     (mouse N-CAM), D-2, 224-1A6-A1, L1-CAM, NILE, Nr-CAM, TAG-1 (axonin-1),  
4     Ng-CAM and F3/F11.

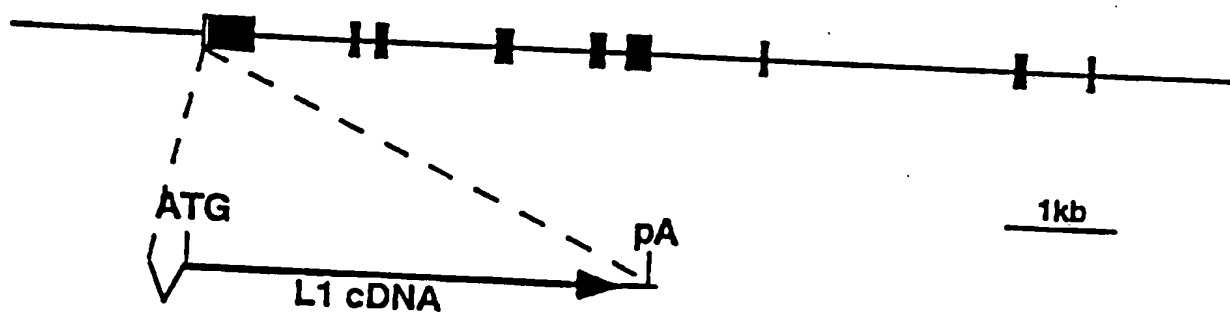
1     39.     The assay system of Claim 36, wherein the neural adhesion molecule is L1.

1     40.     The assay system of Claim 37, wherein the neural adhesion molecule is  
2     selected from the group consisting of laminin, fibronectin, N-cadherin, BSP-2  
3     (mouse N-CAM), D-2, 224-1A6-A1, L1-CAM, NILE, Nr-CAM, TAG-1 (axonin-1),  
4     Ng-CAM and F3/F11.

1     41.     The assay system of Claim 37, wherein the neural adhesion molecule is L1.

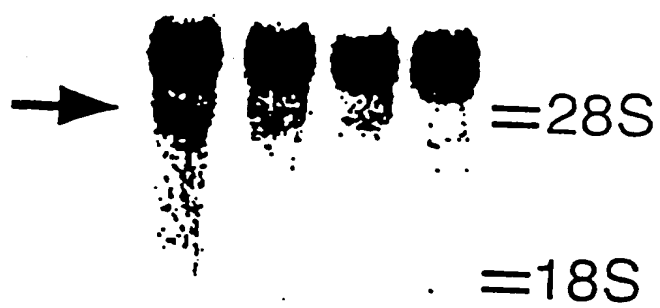
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Figure 1



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Figure 2



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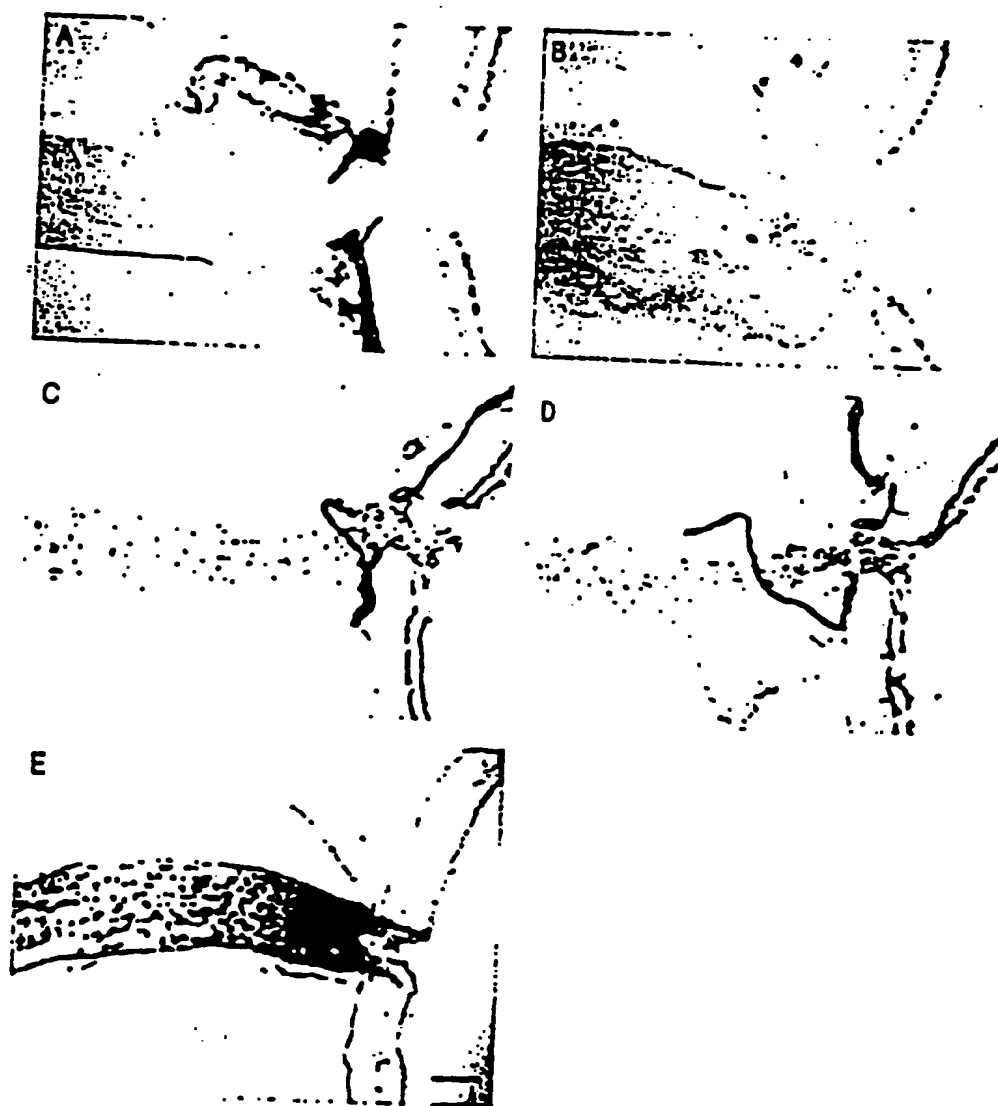
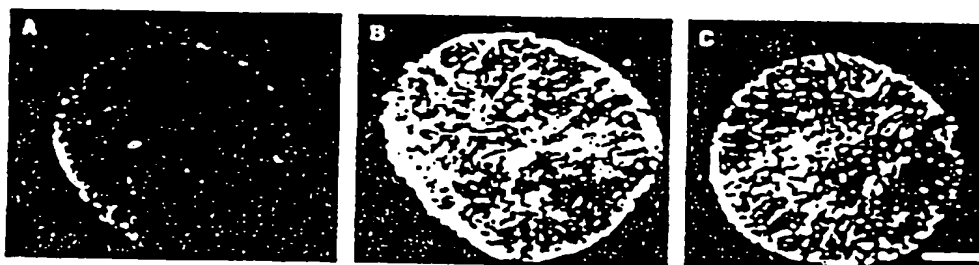
*Figure 3*

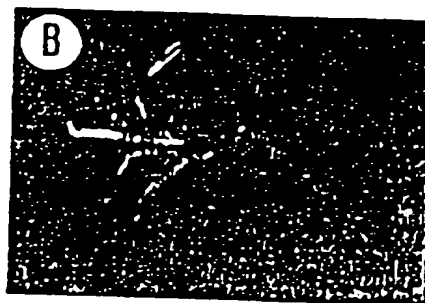
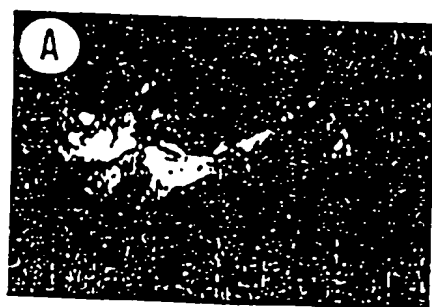


Figure 4

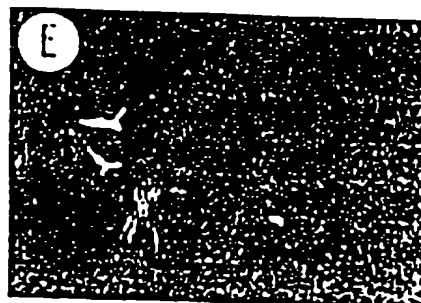
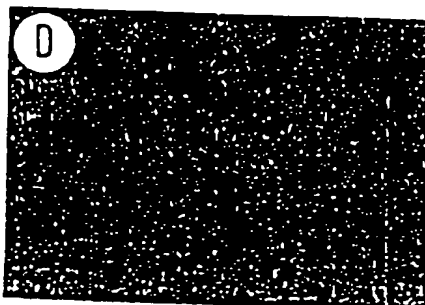


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Figure 5

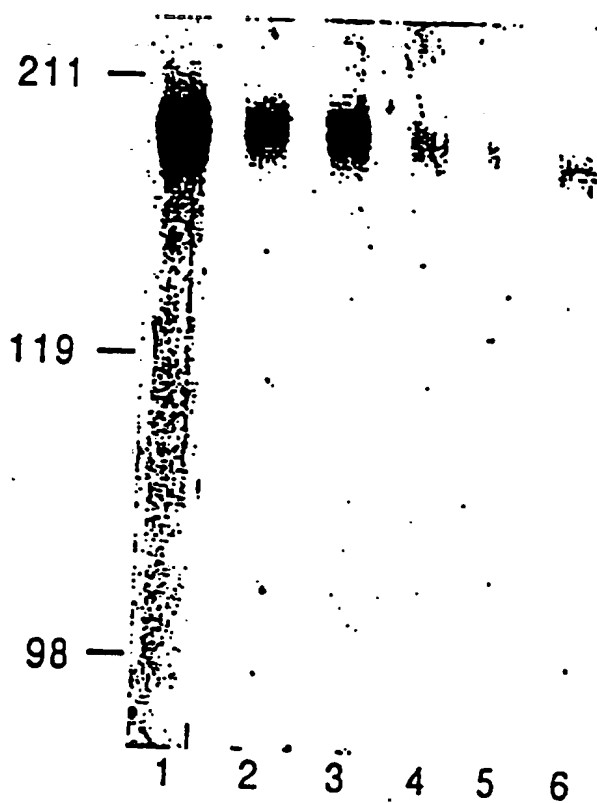


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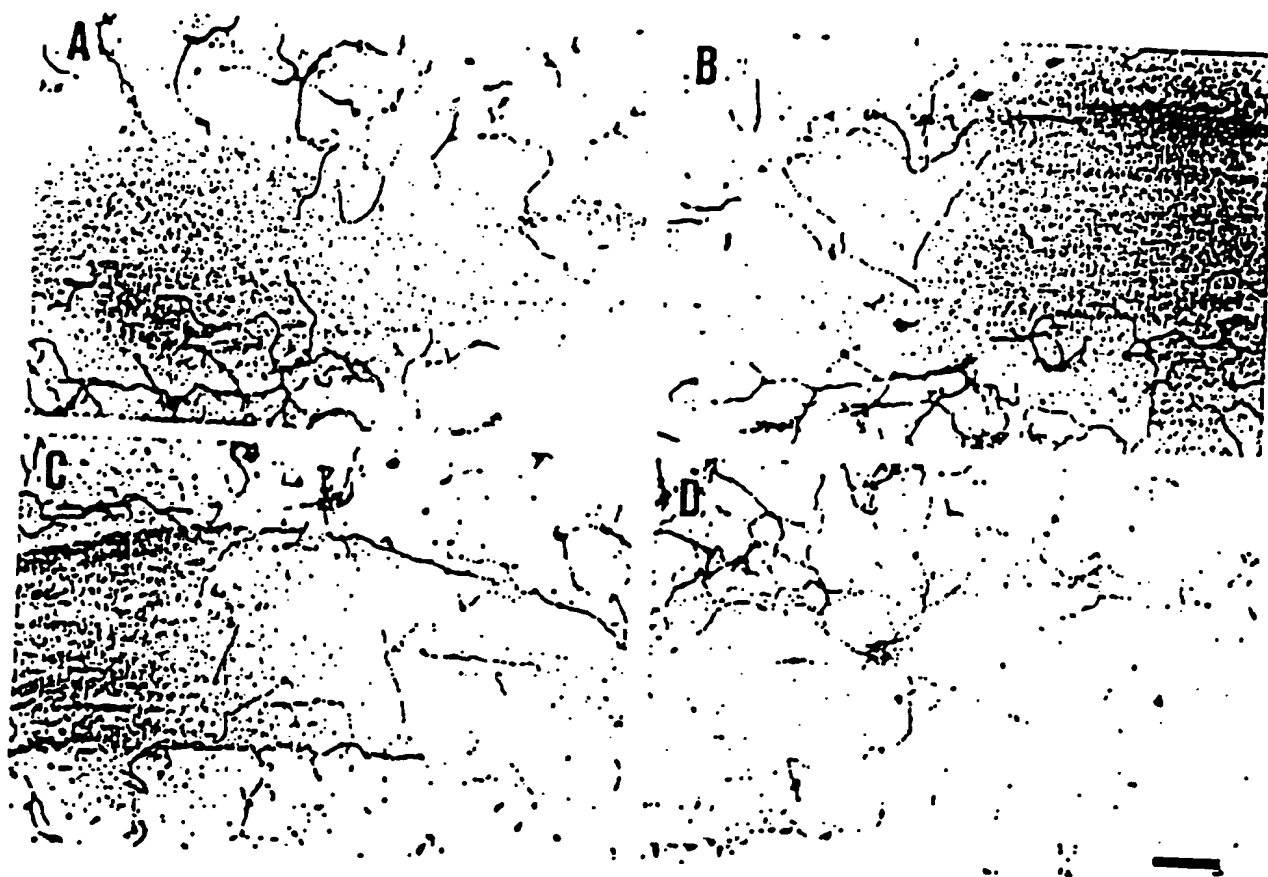
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*Figure 6*

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Figure 7



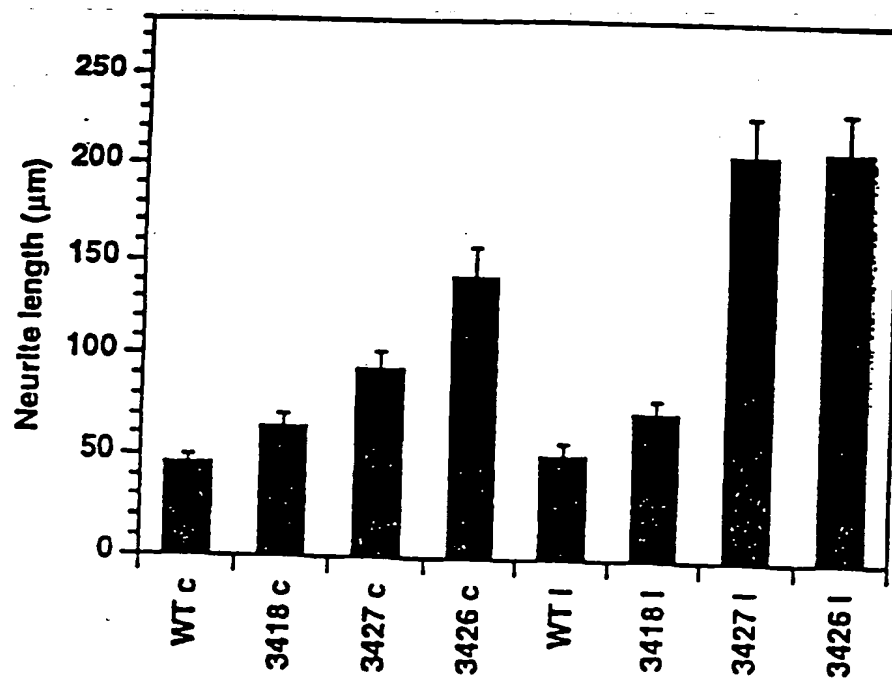
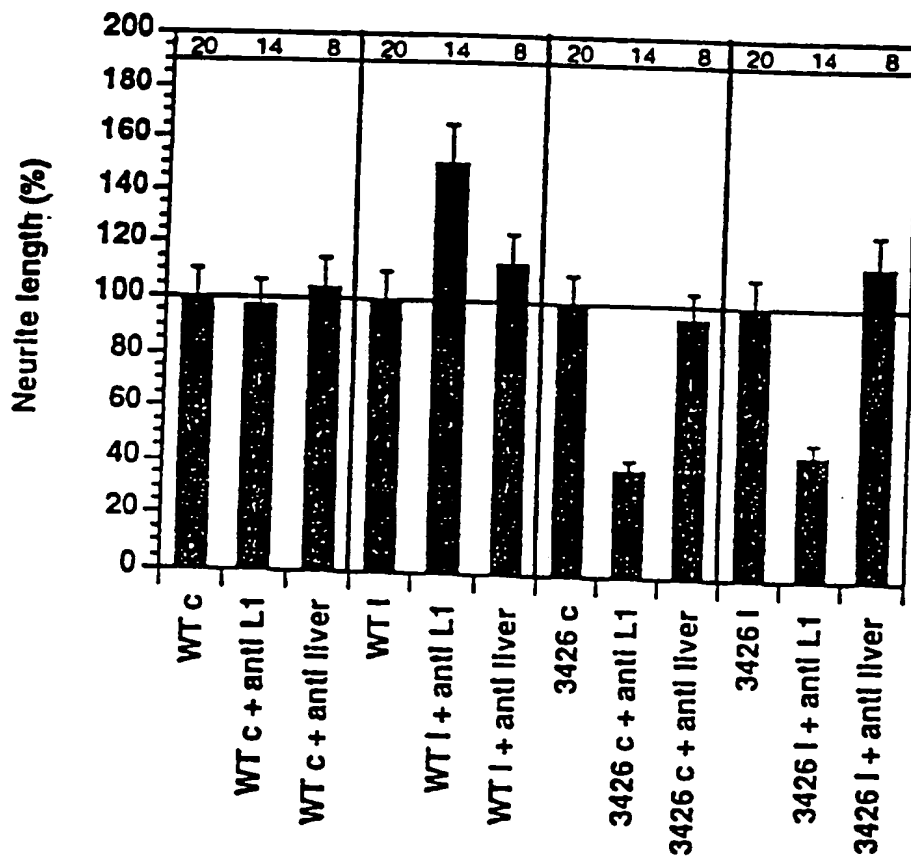
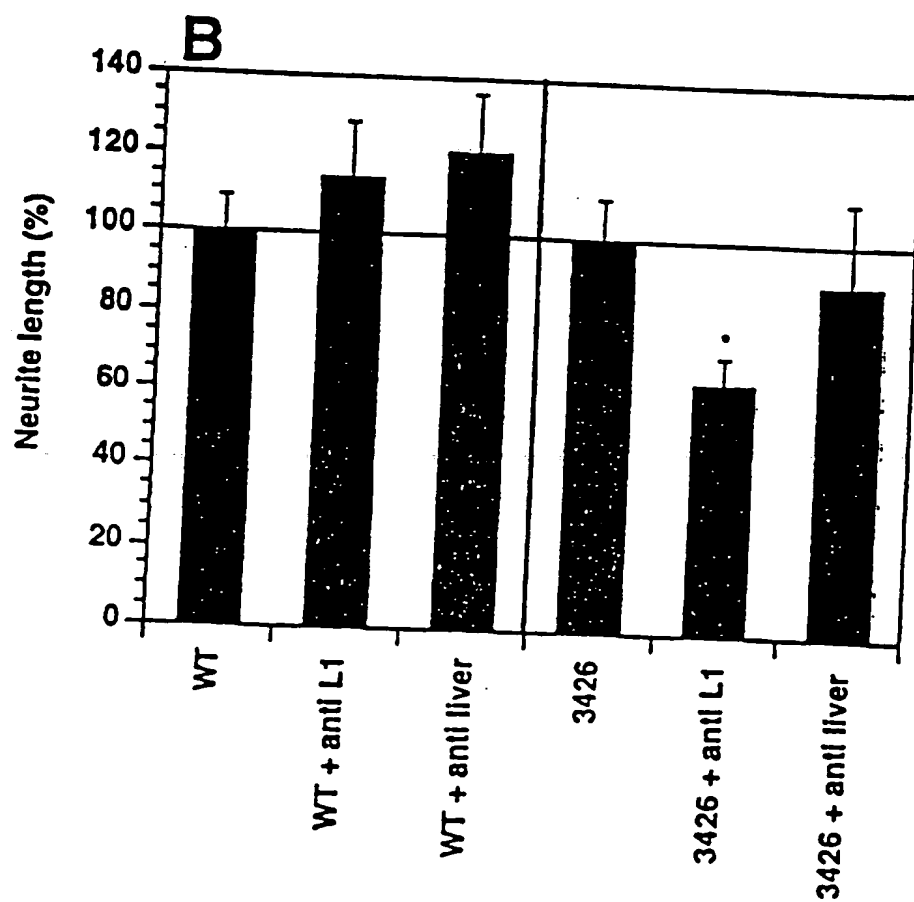
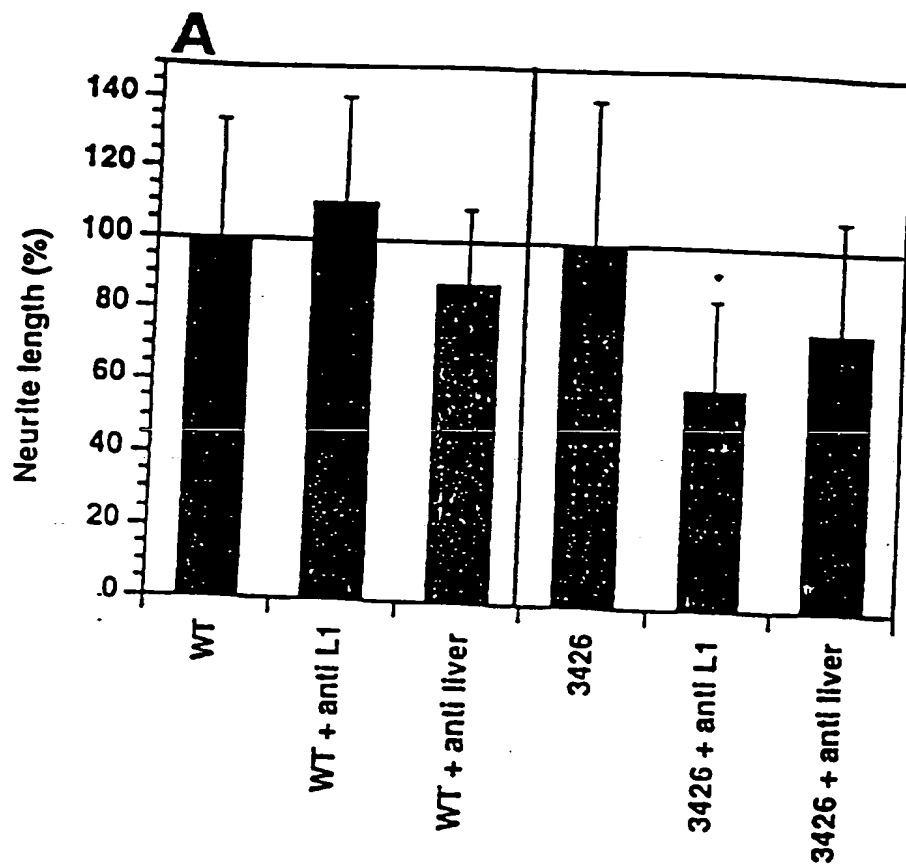
*Figure 8*

Figure 9





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Figure 11

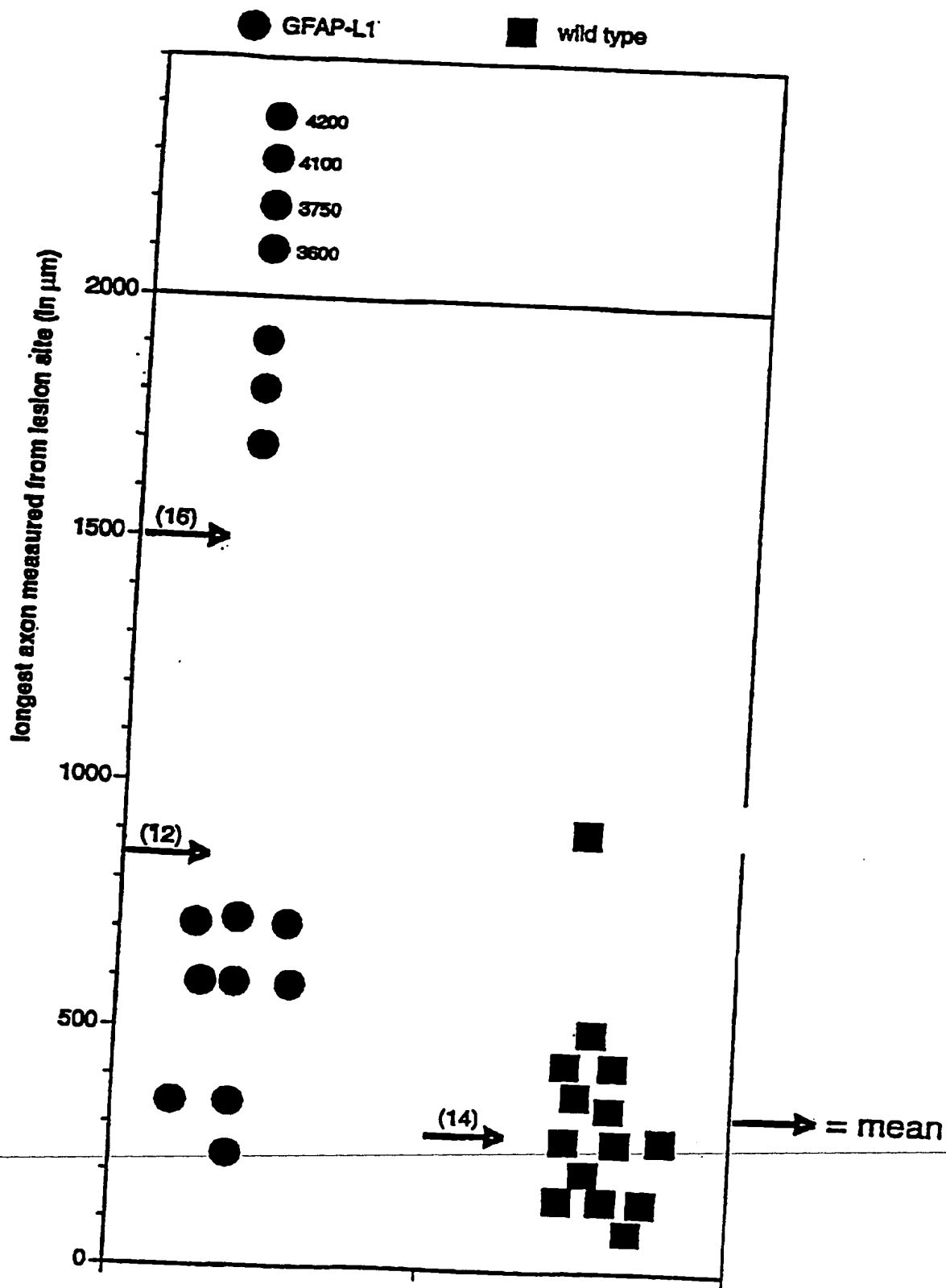
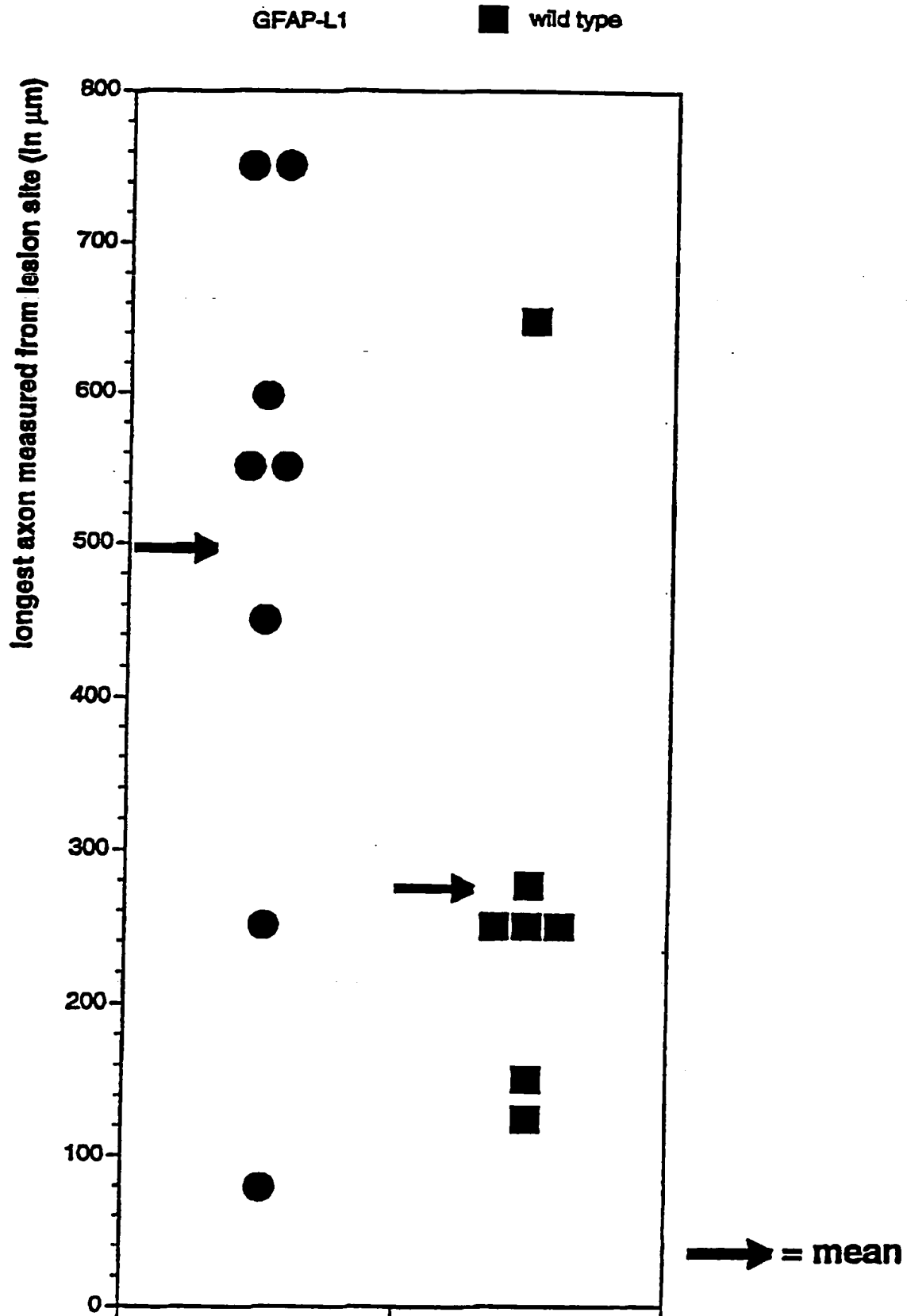




Figure 12



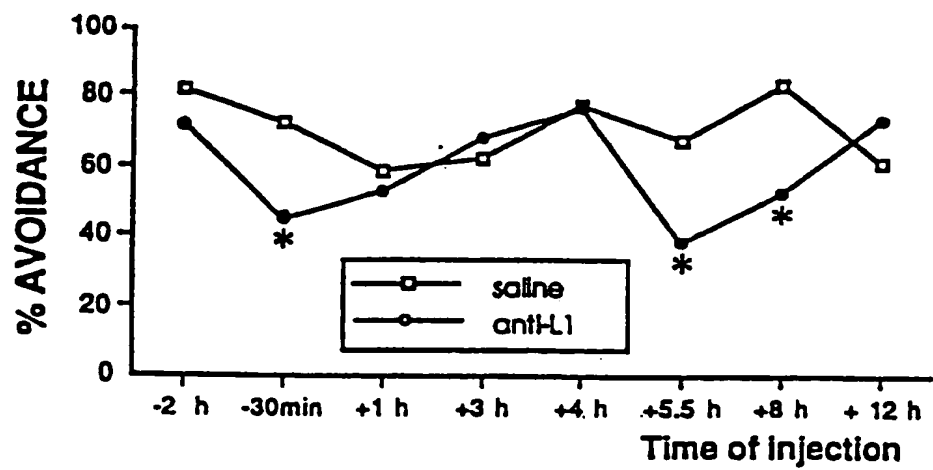
*Figure 13*

Figure 14

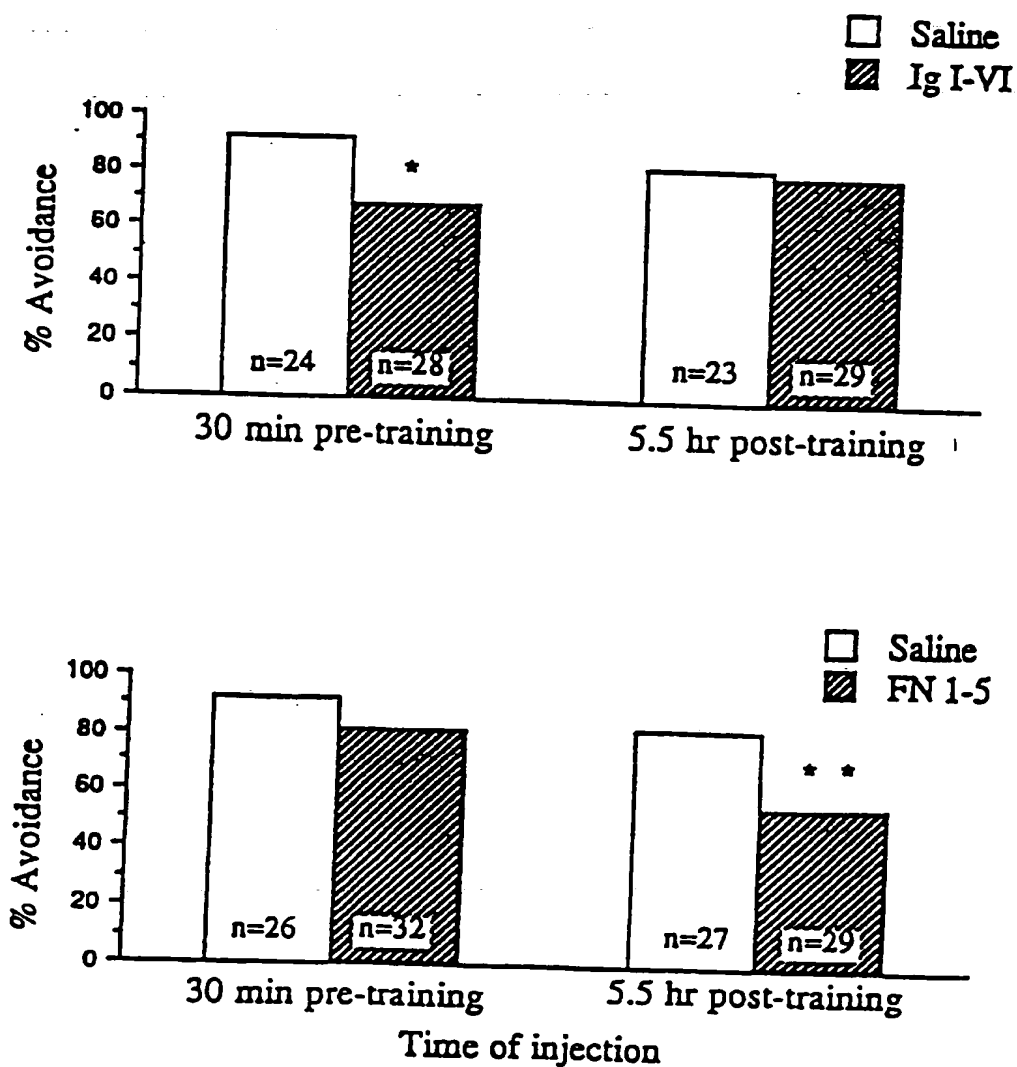


Figure 3. Amnestic effect of L1 fragments

Figure 15

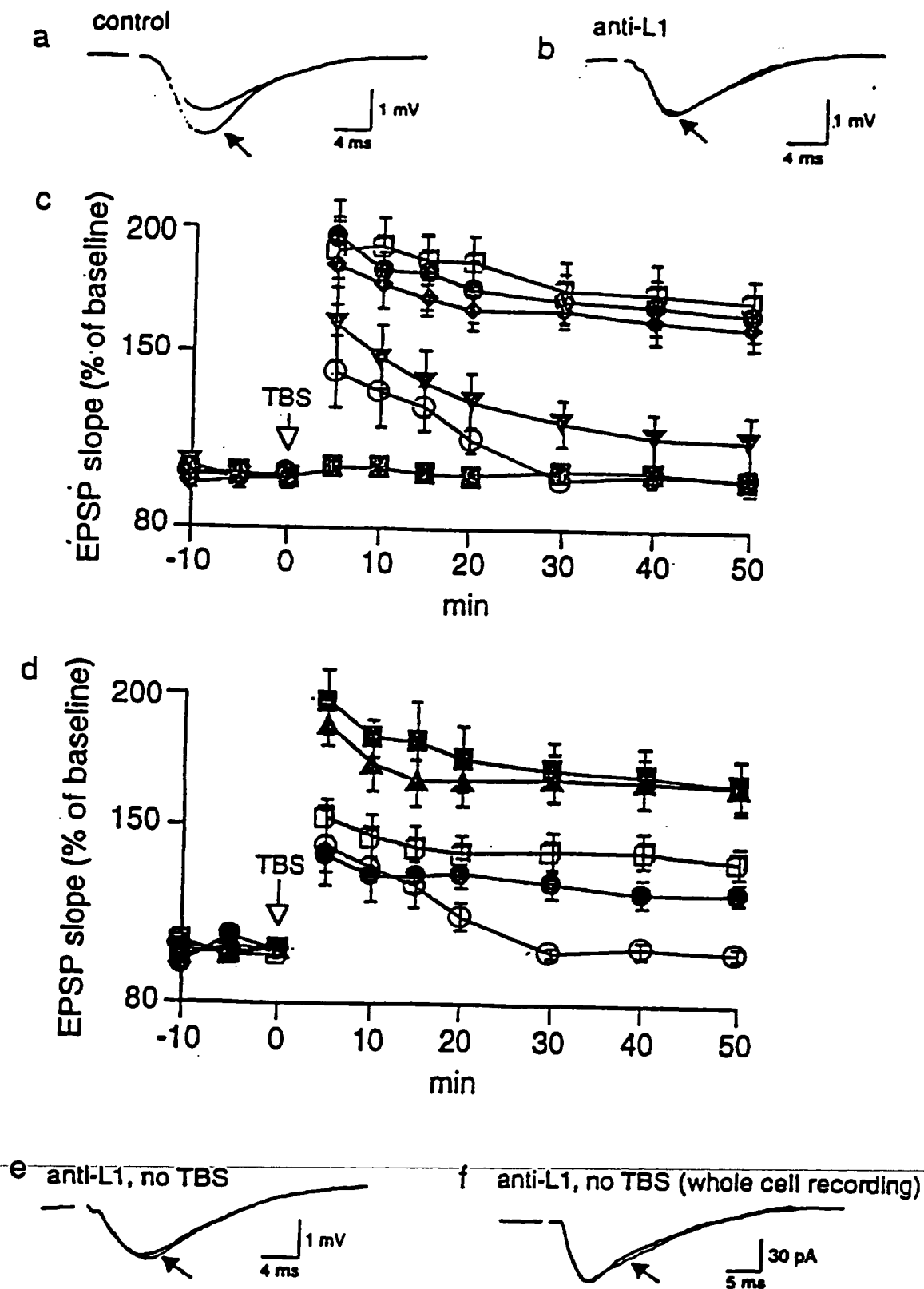


Figure 16

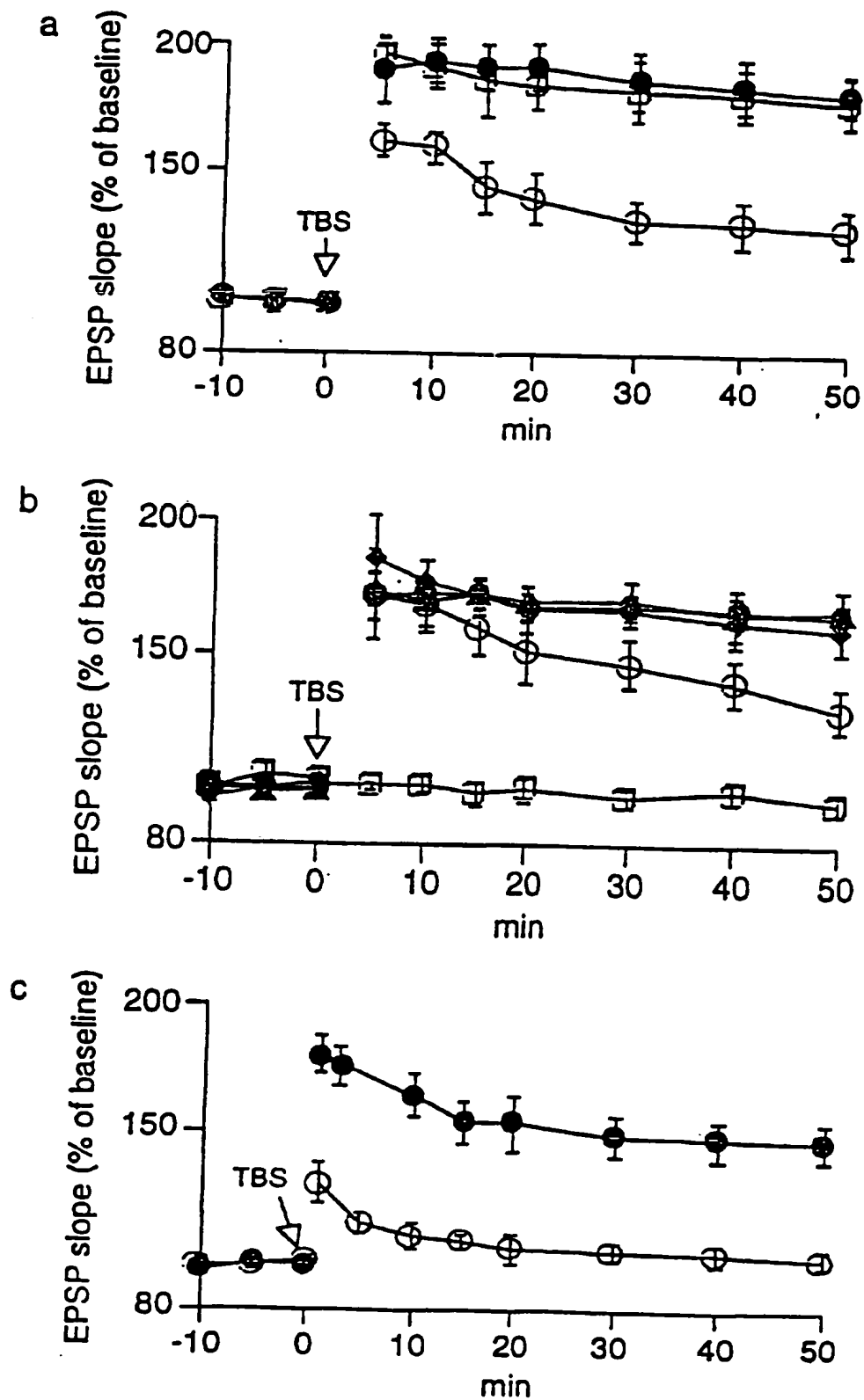
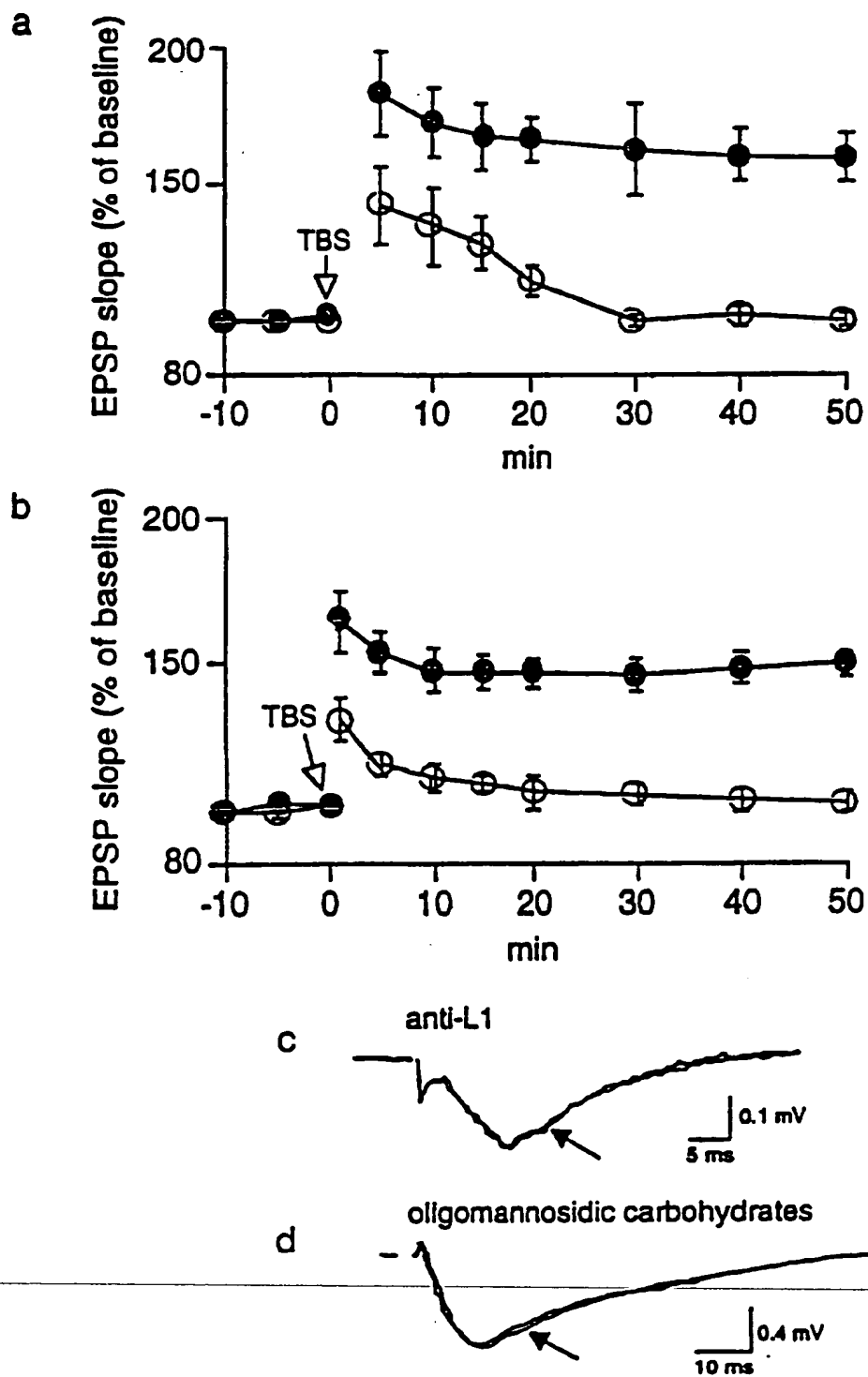
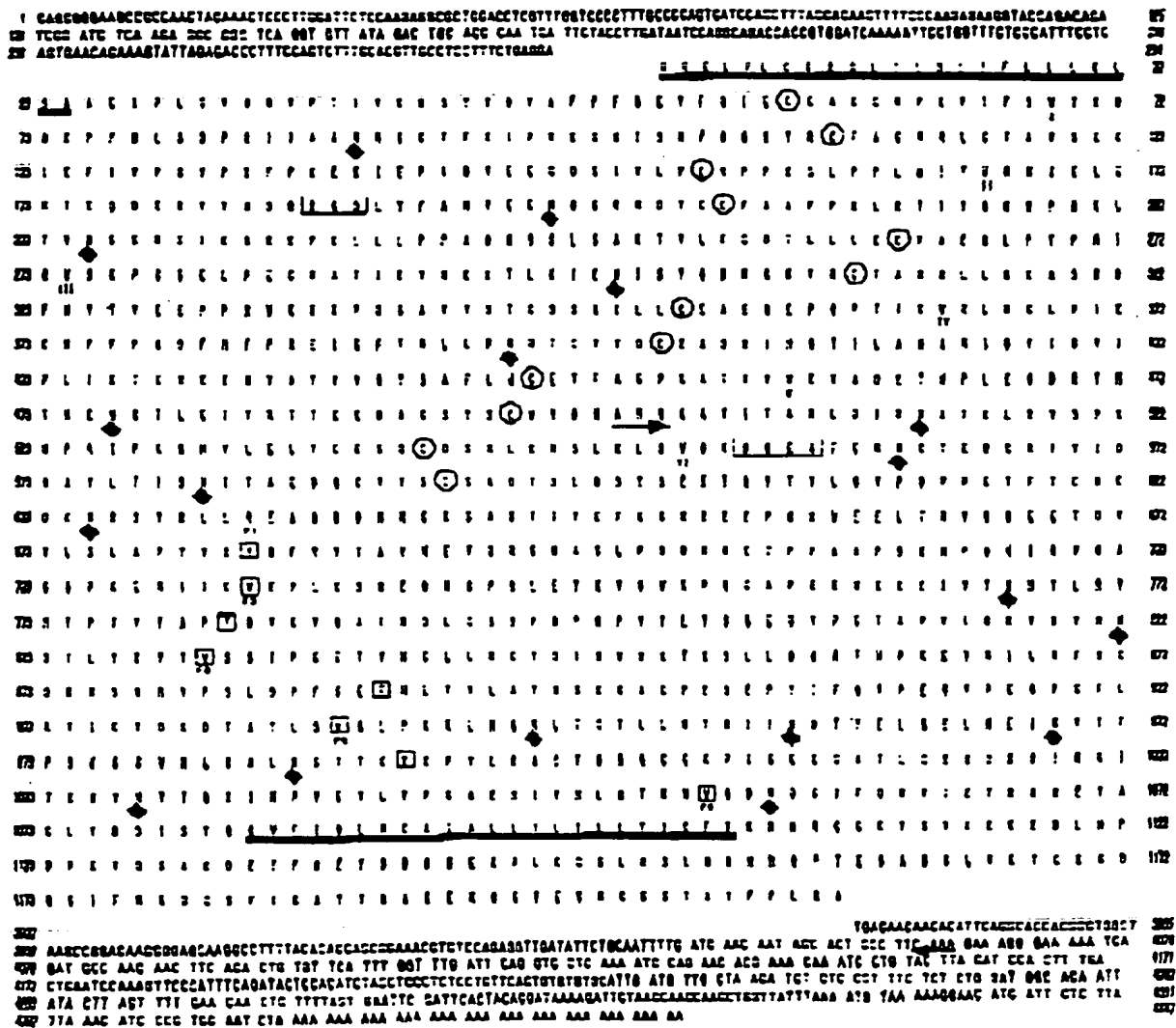


Figure 17



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## FIGURE 18



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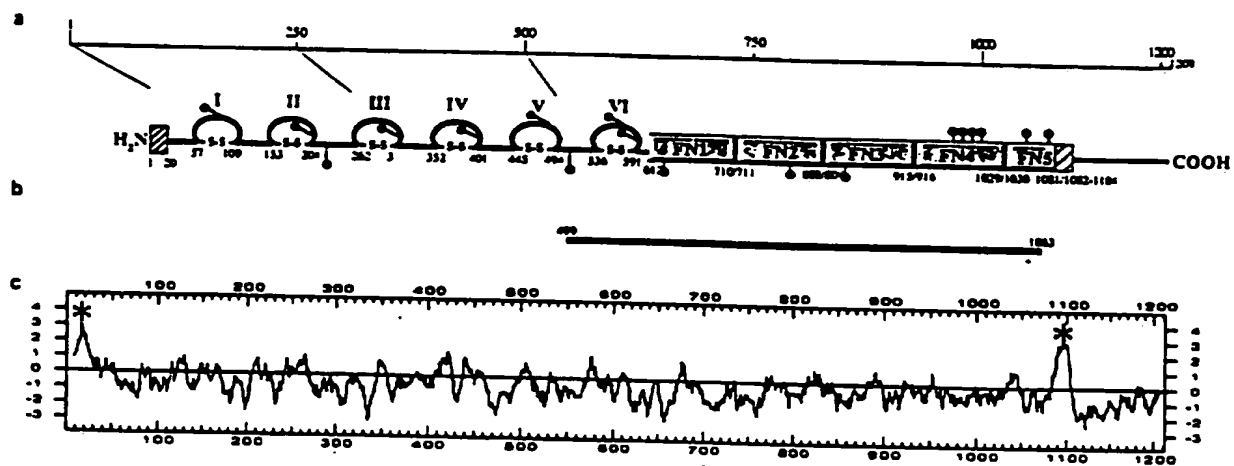


FIGURE 19



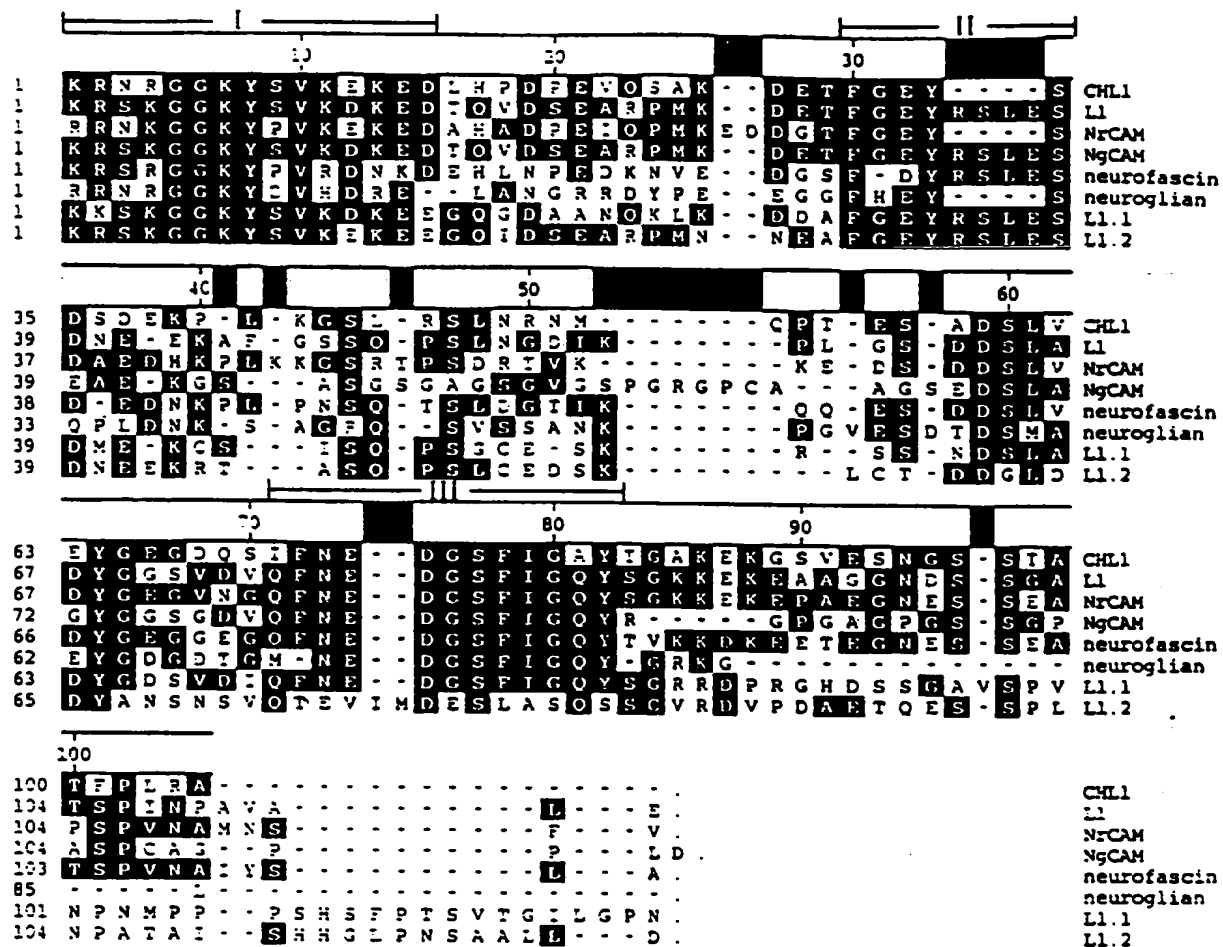


FIGURE 20

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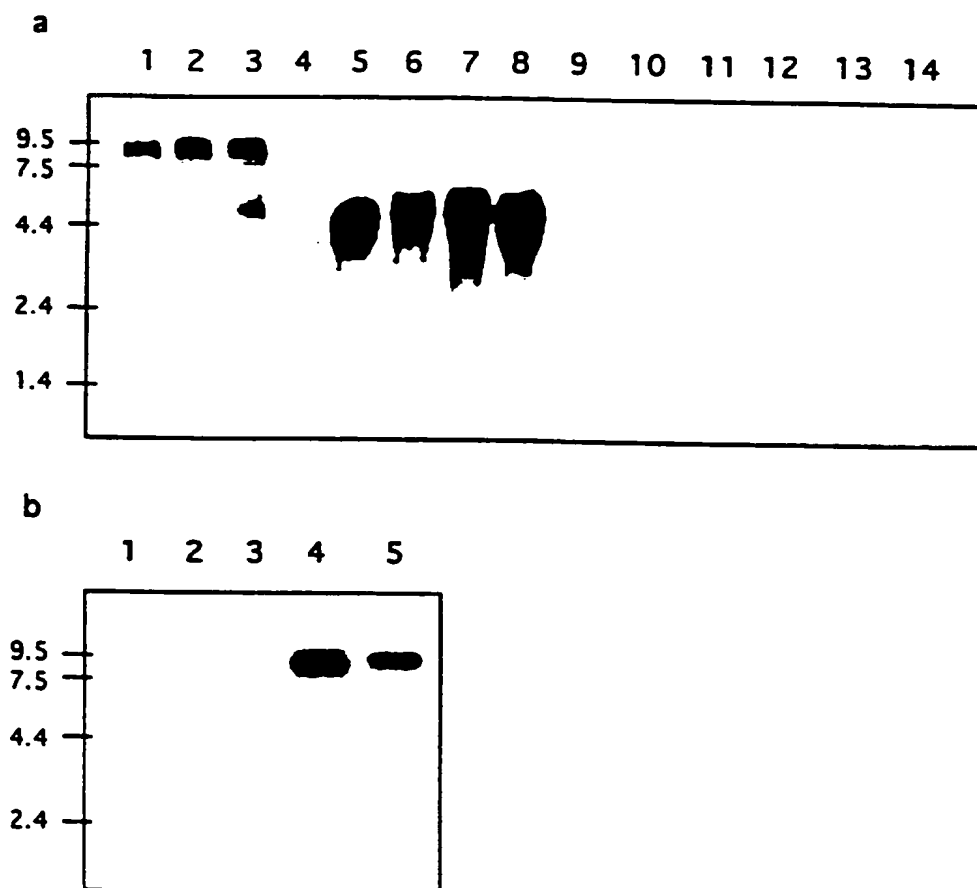


FIGURE 21

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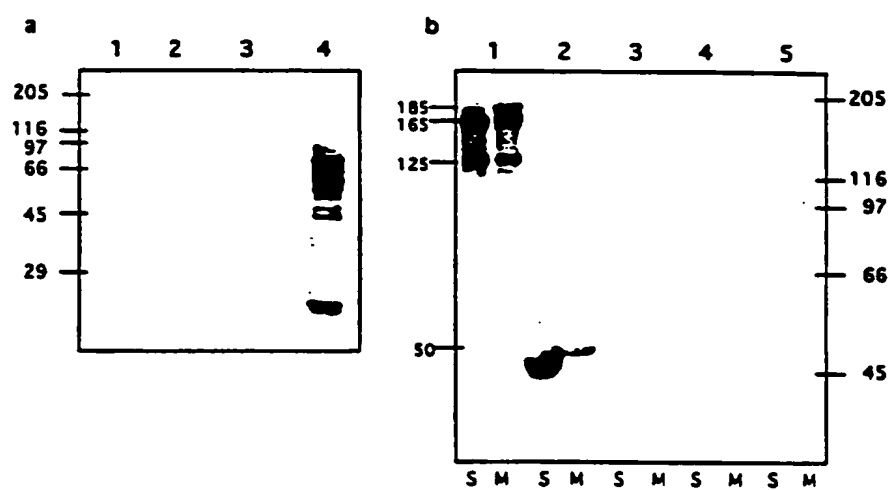


FIGURE 22

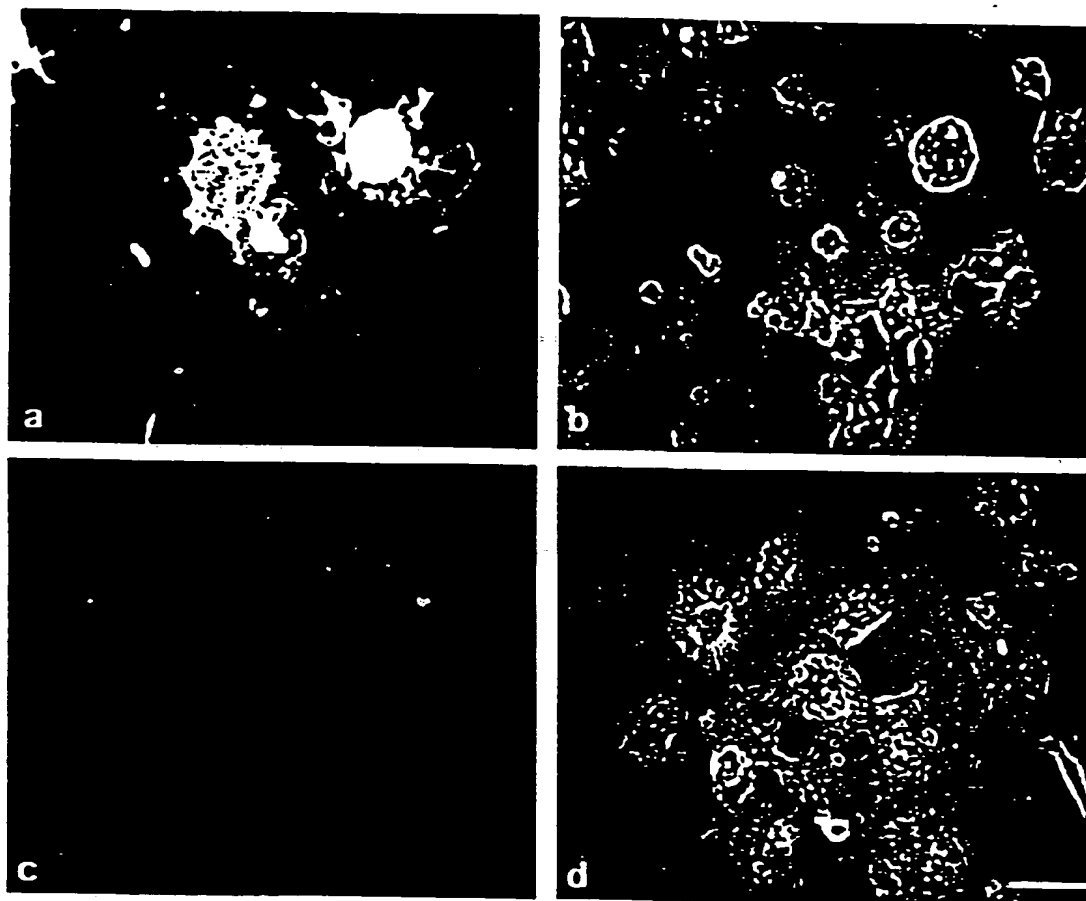


FIGURE 23

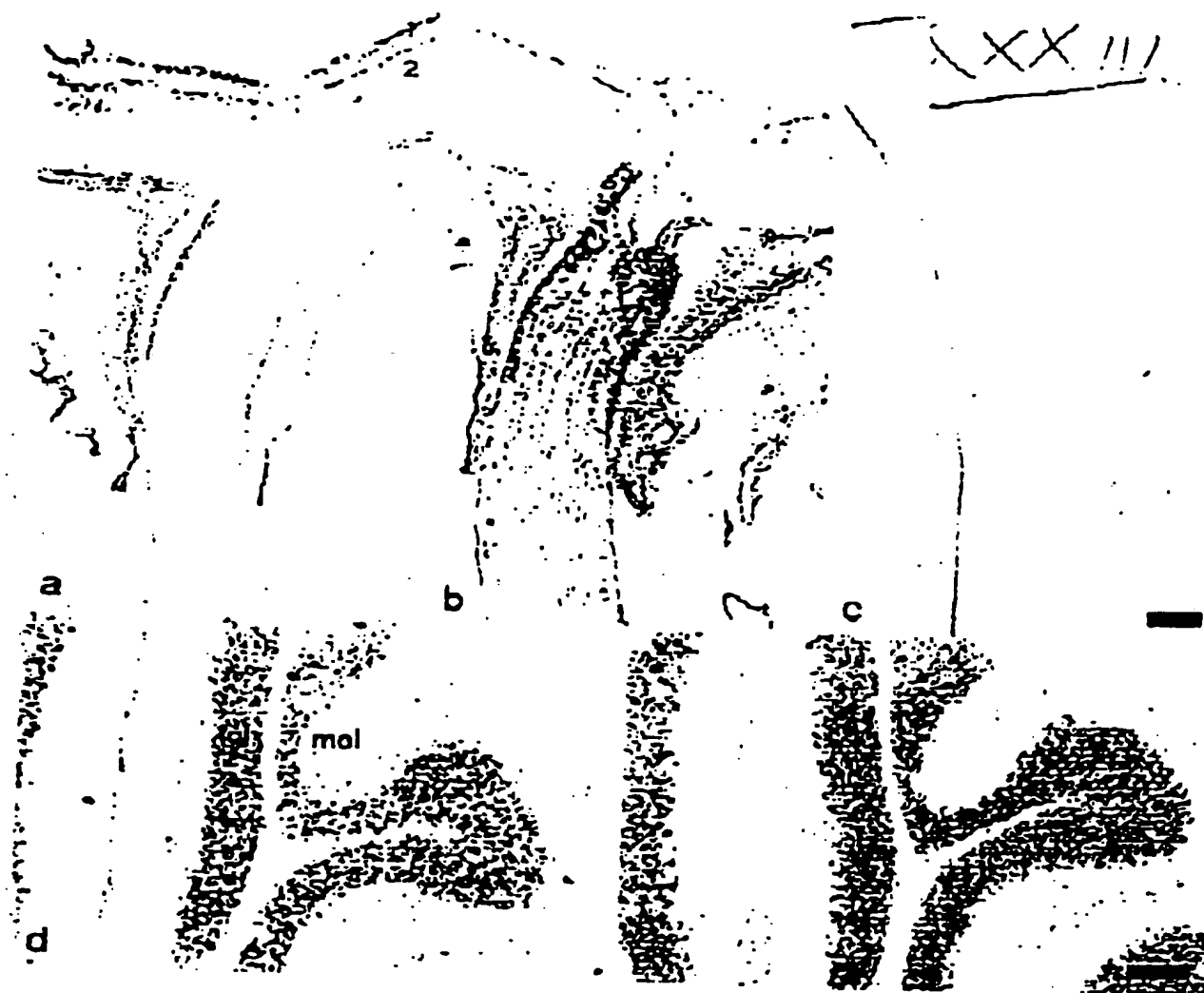


FIGURE 24

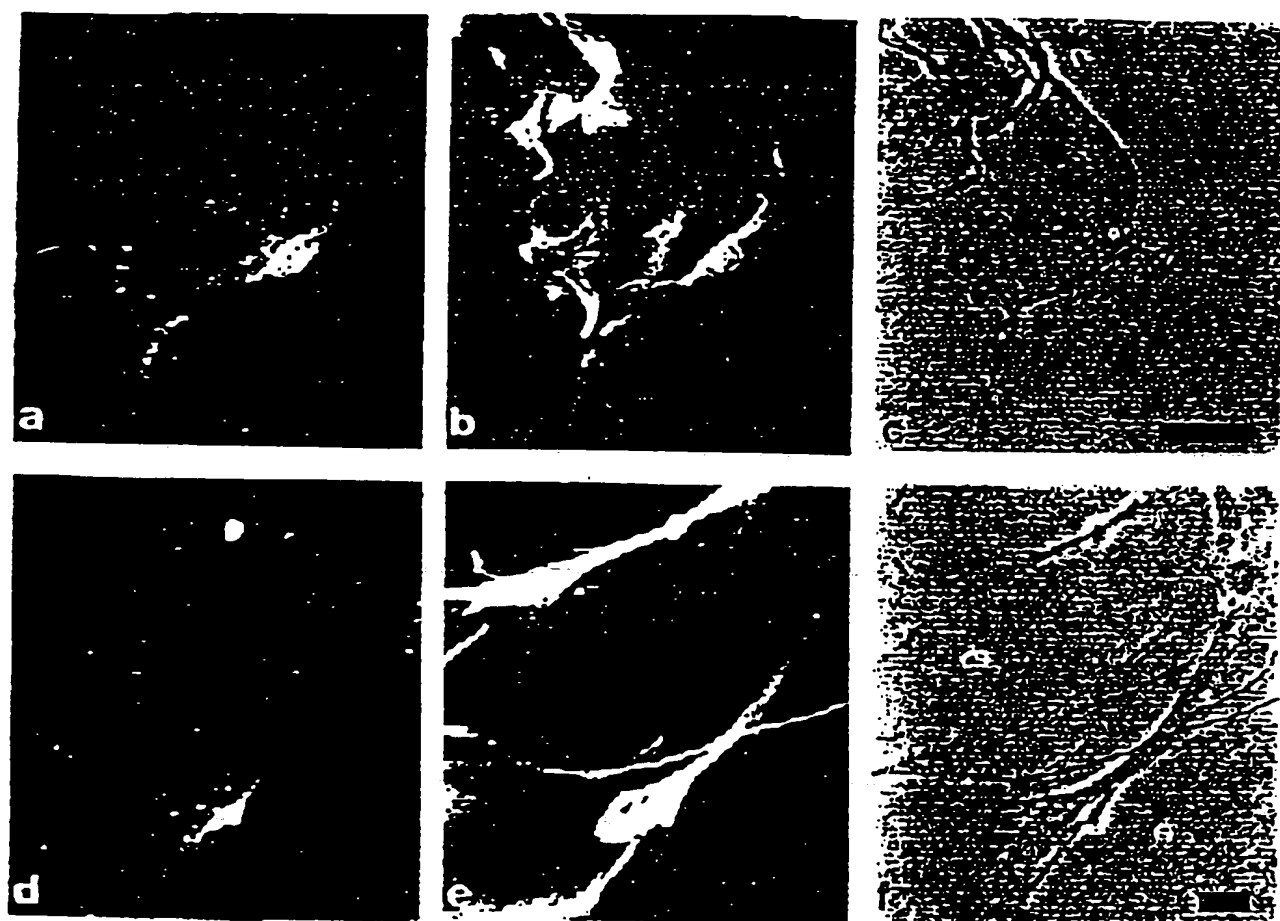


FIGURE 25

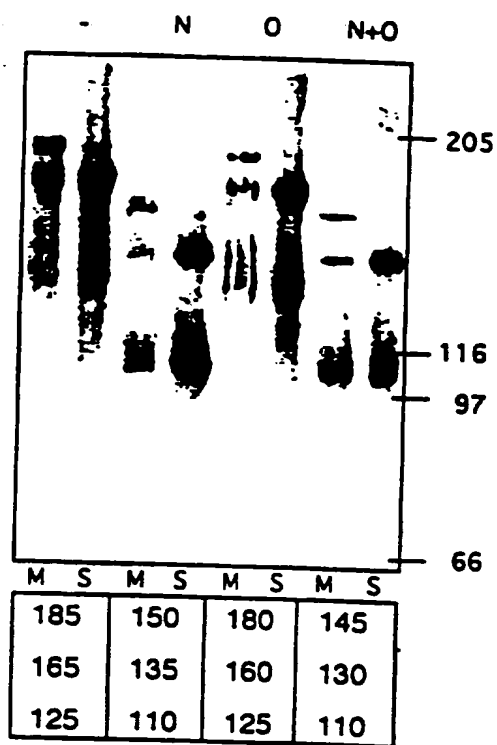


FIGURE 26

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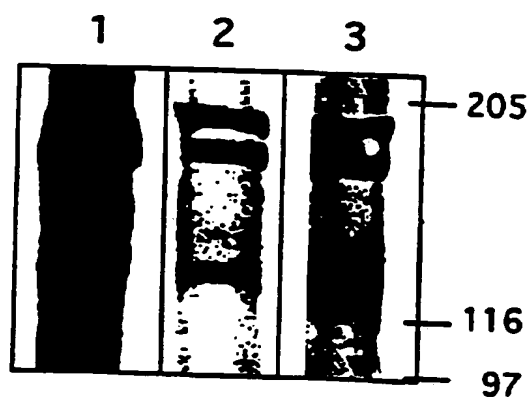


FIGURE 27



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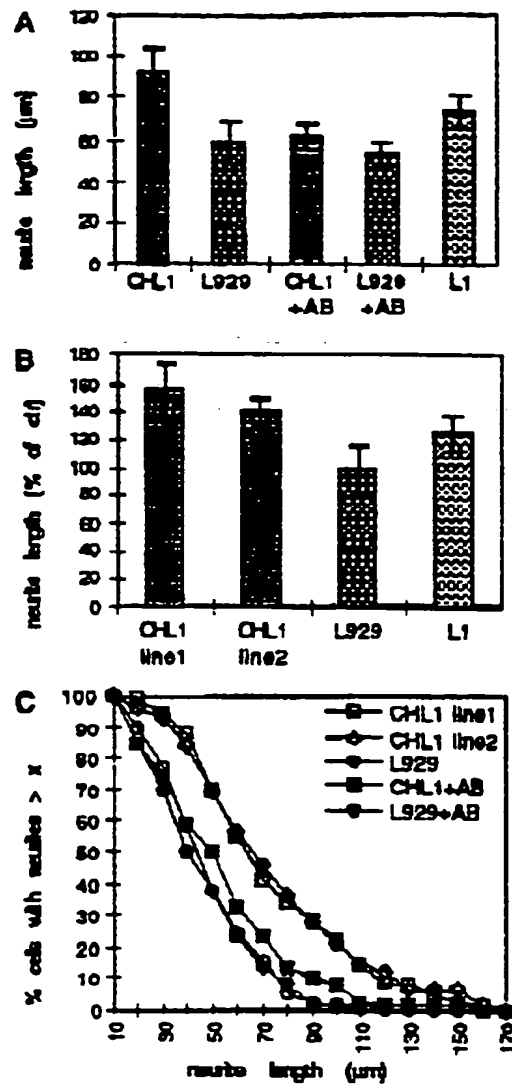


FIGURE 28

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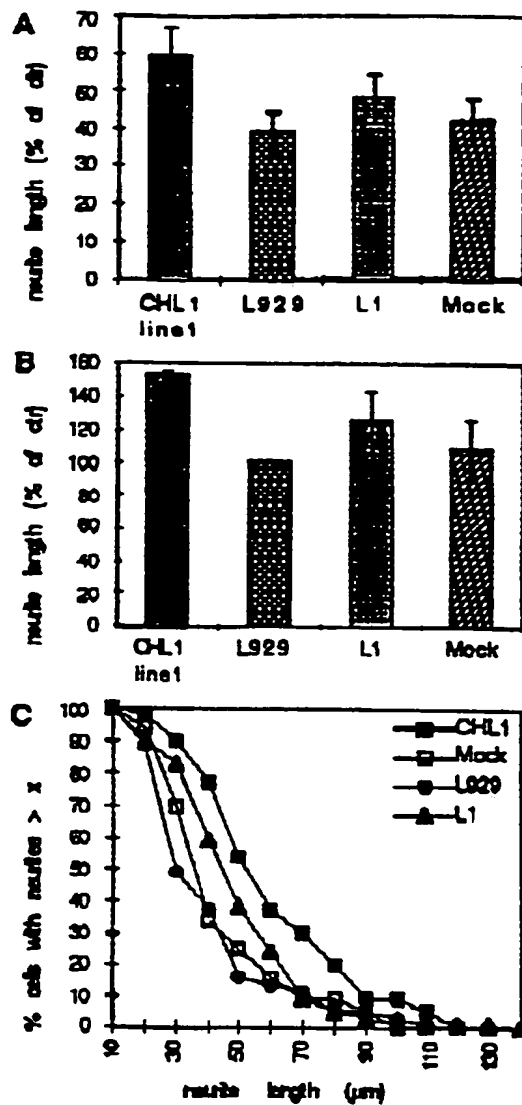


FIGURE 29

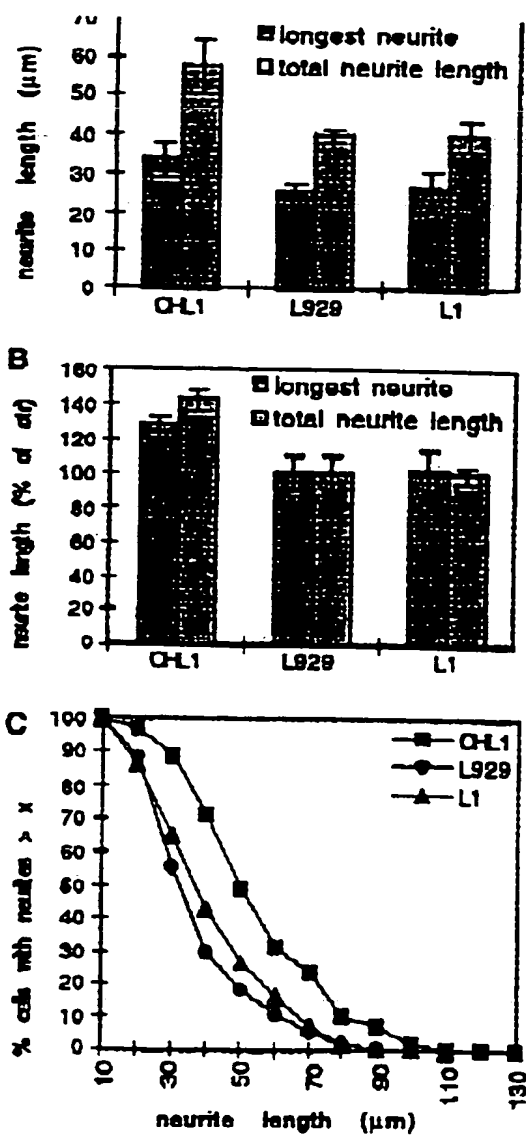


FIGURE 30

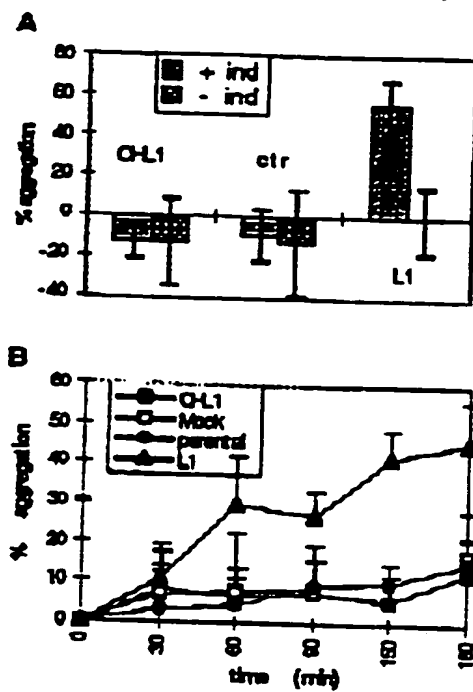


FIGURE 31

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/05434

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/17 A61K39/395 A61K38/39 C12N15/12 C12N5/10  
C07K16/28 A01K67/027 G01N33/50 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07K C12N A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| X,P        | WO,A,95 13291 (UNIV NEW YORK) 18 May 1995<br>see abstract; claims 1-15   | 1-6,14                |
| X,P        | NEUROSCIENCE LETTERS,<br>vol. 188, no. 3, 1995,<br>pages 191-194, XP002009834<br>KOBAYASHI, S. ET AL.: "Grafts of<br>genetically-modified fibroblasts<br>expressing neural cell-adhesion molecule<br>L1 into transfected spinal-cord of adult<br>rats"<br>see the whole document | 7-9,24,<br>28         |
| X          | WO,A,90 05191 (ERZIEHUNGSDIREKTION OF THE<br>CANTON ZURICH) 17 May 1990  | 10-13                 |
| Y          | see page 25 - page 46; claims<br>1-4,24-36,58-77   | 1,7-9,14              |

-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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Date of the actual completion of the international search

31 July 1996

Date of mailing of the international search report

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Authorized officer

Olsen, L

# INTERNATIONAL SEARCH REPORT

Inter national Application No  
PCT/US 96/05434

| C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |   |                       |
|---|---|-----------------------|
| Category  | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
| X   | MOLECULAR AND CELLULAR NEUROSCIENCES,<br>vol. 5, no. 1, 1994,<br>pages 63-77, XP002009835<br>MITTAL, B. AND S. DAVID: "A monoclonal<br>antibody that recognizes an adhesion<br>molecule expressed by certain cells of<br>neuroectodermal and mesenchymal origin"<br>see the whole document                            | 10,12,13              |
| Y   | ---   | 1-6,14                |
| X   | THE EMBO JOURNAL,<br>vol. 3, no. 1, 1984,<br>pages 1-10, XP002009836<br>RATHJEN, F.G. AND M. SCHACHNER:<br>"Immunocytological and biochemical<br>characterization of a new neural cell<br>surface component (L1 antigen) which is<br>involved in cell adhesion"<br>cited in the application<br>see the whole document | 10-13                 |
| Y   | ---   | 1-6                   |
| Y   | NEURON,<br>vol. 14, January 1995,<br>pages 57-66, XP002009837<br>DOHERTY P. ET AL: "A soluble chimeric<br>form of the L1 glycoprotein stimulates<br>neurite outgrowth"<br>cited in the application<br>see the whole document  | 1-9,14                |
| Y   | ---   |                       |
| Y   | THE JOURNAL OF NEUROSCIENCE,<br>vol. 13, no. 11, 1993,<br>pages 4764-4775, XP002009838<br>APPEL, F. ET AL.: "Several extracellular<br>domains of the neural cell adhesion<br>molecule L1 are involved in neurite<br>outgrowth and cell body adhesion"<br>cited in the application<br>see the whole document           | 1-9,14                |
| Y   | ---   |                       |
| Y   | THE JOURNAL OF CELL BIOLOGY,<br>vol. 119, no. 4, 1992,<br>pages 883-892, XP002009839<br>WILLIAMS, E.J. ET AL.: "Calcium influx<br>into neurons can solely account for cell<br>contact-dependent neurite outgrowth<br>stimulated by transfected L1"<br>cited in the application<br>see the whole document              | 1-9                   |
|   | ---   |                       |
|   | -/--  |                       |

# INTERNATIONAL SEARCH REPORT

Inter      nal Application No  
PCT/US 96/05434

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|----------|---|-----------------------|
| Y        | BRAIN RESEARCH,<br>vol. 619, 1993,<br>pages 255-262, XP002009840<br>POLTORAK, M. ET AL.: "Degradation<br>fragments of L1 antigen enhance tyrosine<br>hydroxylase-immunoreactive neurite<br>outgrowth in mesencephalic cell culture"<br>see the whole document<br>---  | 1-6,14                |
| Y        | WO,A,93 00427 (ERZIEHUNGSDIREKTION OF THE<br>CANTON OF ZURICH) 7 January 1993<br>see page 26 - page 51; claims 1-4,24-27<br>---   | 1,7-9,14              |
| A        | GLIA,<br>vol. 10, 1994,<br>pages 70-74, XP002009841<br>MARTINI, R. ET AL.: "restricted<br>localization of L1 and N-CAM at sites of<br>contact between Schwann cells and neurites<br>in culture"<br>cited in the application<br>see the whole document<br>---  | 1-41                  |
| A        | CURRENT OPINION IN NEUROBIOLOGY,<br>vol. 4, 1994,<br>pages 726-734, XP002009842<br>SCHACHNER, M.: "Neural recognition<br>molecules in disease and regeneration"<br>see the whole document<br>---  | 1-41                  |
| A        | THE JOURNAL OF NEUROSCIENCE,<br>vol. 13, no. 5, 1993,<br>pages 2217-2229, XP002009843<br>POLTORAK, M. ET AL.: "Effects of frontal<br>cortical lesions on mouse stratum:<br>reorganization of cell recognition<br>molecule, glial fiber, and synaptic<br>protein expression in the dorsomedial<br>striatum"<br>see the whole document<br>----- | 1-41                  |

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/05434

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)  | Publication<br>date  |
|---|---------------------|---|--|
| WO-A-9513291                              | 18-05-95            | AU-B- 1091495<br>CA-A- 2173731  | 29-05-95<br>18-05-95   |
| WO-A-9005191                              | 17-05-90            | AT-T- 124726<br>AU-B- 652537<br>AU-B- 4528089<br>AU-B- 7435494<br>DE-D- 68923362<br>DE-T- 68923362<br>EP-A- 0396719<br>JP-A- 4117298<br>US-A- 5250414 | 15-07-95<br>01-09-94<br>28-05-90<br>15-12-94<br>10-08-95<br>28-03-96<br>14-11-90<br>17-04-92<br>05-10-93 |
| WO-A-9300427                              | 07-01-93            | US-A- 5250414<br>AU-B- 2152092  | 05-10-93<br>25-01-93   |